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Identification of *Vibrio cholerae* by Pyrolysis Gas-Liquid Chromatography

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Cholera-like vibrios examined by pyrolysis gas-liquid chromatography could be distinguished from other common aerobic gram-negative bacilli, including oxidase-positive organisms, e.g., *Aeromonas*. Vibrios in Heiberg group I were subdivided into three types on the basis of differences in one complex in the chromatogram, and these closely corresponded with the identification as classical, El Tor, or "intermediate" biotypes of *Vibrio cholerae* by conventional methods.

Reiner (4) used pyrolysis gas-liquid chromatography (PGLC) of whole bacteria as a means of identification and showed that it was possible to differentiate between species of *Mycobacterium* and even between serotypes of *Escherichia coli*. Myers and Watson (3) used PGLC for the rapid diagnosis of viral and fungal diseases of plants, whereas Vincent and Kulik (6) have applied it to the subdivision of fungi of the *Aspergillus flavus* group.

The present cholera pandemic has re-emphasised the difficulties which may be encountered in differentiating vibrios from other oxidase-positive gram-negative bacilli, and also the deficiencies of present-day techniques in accurately subdividing the varieties of *Vibrio cholerae*. For this reason we believed that PGLC might be a useful addition to the methods currently available.

MATERIALS AND METHODS

Organisms. Cholera-like vibrios (57 strains) were obtained from a variety of sources: 1 strain from the National Collection of Type Cultures (NCTC); 4 strains from the Manchester University Collection of Bacteria (MUCOB); 3 strains from Glaxo Laboratories; 5 recent isolates from Singapore; 11 from Ibadan; and also 33 strains from Calcutta, 31 of which had been collected over the last 20 years and 2 which were reference strains. Other bacteria included strains from NCTC and from MUCOB and recent isolates, from human sources, of *Vibrio parahaemolyticus*, 2; *V. proteus*, 1; *Salmonella* sp., 2; *E. coli*, 7; *Shigella* sp., 3; *Proteus* sp., 2; *Aeromonas* sp., 11; and *Pseudomonas aeruginosa*, 2.

Preparation of bacteria for PGLC. Each strain was grown at 37 C for 20 hr in a 50-ml static culture of nutrient broth (Oxoid CM67). A 1.5-ml amount of Formalin (40% formaldehyde) was added, and after 20 min the bacteria were harvested by centrifugation, washed three times, and finally resuspended in 0.2 ml of distilled water.

Chromatographic methods. Analyses were performed on a Pye "Series 104" gas chromatograph with pyrolysis unit and dual stainless-steel columns (⅛ inch diameter, 12 feet long; ca. 0.3 cm diameter, ca. 3.7 m long) packed with 20 M polyethylene glycol. The nitrogen carrier was adjusted to 42 psi gauge with a flow rate of 20 ml per min. The oven was temperature programmed to increase from 50 to 145 C at a rate of 12 C per min and was then maintained at this temperature until the analysis was complete, about 24 min after firing. The sensitivity of the dual hydrogen flame detector was set at 2×10^{-10} amp, and the recorder was driven at a chart speed of 0.5 inch (ca. 1.27 cm) per min.

Samples were loaded on to the coiled filament by dipping it twice into the bacterial suspension and drying it after each occasion in a hot air blower. The sample was pyrolyzed at a firing temperature of 800 C for 5 sec. Separate suspensions were made and analyzed from duplicate cultures of each strain of bacterium, and in many cases a second sample was tested from one of these two bacterial suspensions.

Conventional tests to differentiate the biotypes of V. cholerae. Hemolysin production was assessed by the method of Greig (2), but sheep erythrocytes, instead of goat, were added to the broth cultures. For the Voges-Proskauer (VP) reaction, the method of Barritt (1) was used, but cultures were grown for 2 days at 30 C instead of 3 days at 37 C. Polymyxin resistance was determined by both the disc method and the plate method of Roy et al. (5), but in each case the inoculum was 1/100 dilution of an overnight broth culture, not an undiluted 2-hr broth culture.

A detailed critical assessment of these three tests is being prepared for publication.

RESULTS AND DISCUSSION

The pyrochromatograms of a vibrio (*V. cholerae*) and a nonvibrio (*Aeromonas* sp.) are

shown in Fig. 1. They may be divided into complexes of individual peaks, identifiable by their retention times (time from firing). The complexes were numbered, and the peaks were lettered. The most significant for identification of vibrios is complex 7, which had a retention time of 6.25 to 7.25 min and consisted of two major peaks α and β, together with two or three minor peaks.

Peak 7β was always present, with nonvibrios and vibrios. With the nonvibrios tested (*Aeromonas*, *Pseudomonas*, *Escherichia*, *Salmonella*, *Shigella*, *Proteus*), peak 7α was either absent or represented by a small notch on the rising curve of 7β. Peak 7α was always present with vibrios. With the three strains of *V. parahaemolyticus* and *V. proteus* that were tested (all indole-negative and cholera-red-negative), 7α was small; but with all the strains of cholera-like vibrios, it was prominent.

PGLC did not distinguish between the various Heiberg groups, nor between agglutinable and nonagglutinable vibrios, nor between different serotypes, but 45 strains of Heiberg group I (40 of which were agglutinable by cholera antiserum) fell into three categories (see Table 1 and Fig. 2).

Category (i). Peak 7α, though prominent, was distinctly smaller than 7β. These 10 strains were found by conventional methods to be *V. cholerae* (classical biotype): hemolysin-negative; VP reaction, negative or very slight; sensitive to polymyxin B (7.5 IU per ml).

Category (ii). Peak 7α was distinctly larger than 7β. This was so with all the five strains of *V. cholerae* (El Tor biotype): hemolysin-positive; moderate or strong VP reaction; resistant to polymyxin (15 IU per ml). It applied also to four strains which by conventional tests were of intermediate biotype (*see below*).

TABLE 1. *Classification of 45 strains of V. cholerae by conventional methods and by PGLC*

Biotype by conventional methods	Category by PGLC		
	Classical-like	El Tor-like	Intermediate
Classical	10		
El Tor		5[a]	
Intermediate		4[b]	26[c]

[a] Two of these five strains were not agglutinable by cholera antiserum.

[b] One of these four strains was not agglutinable by cholera antiserum.

[c] Two of these 26 strains were not agglutinable by cholera antiserum.

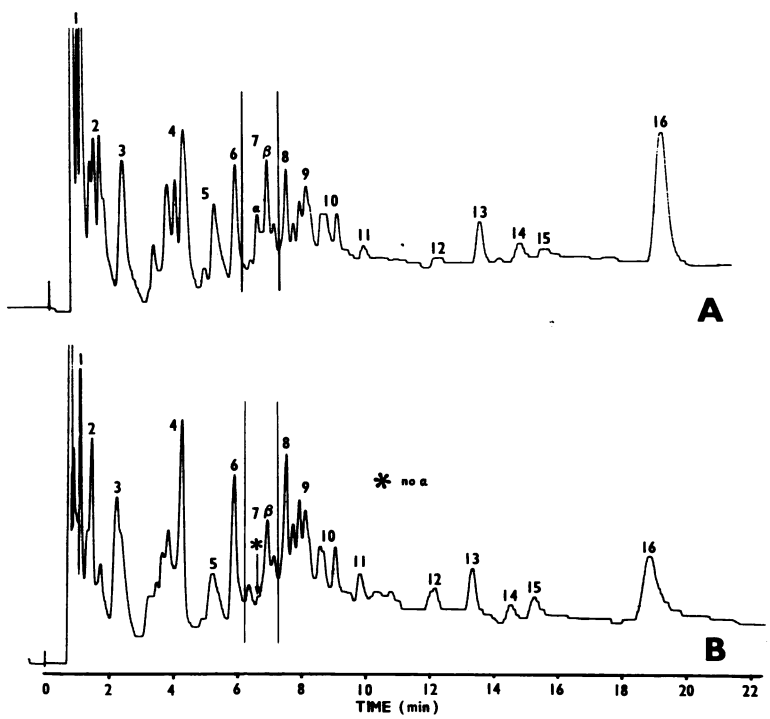

FIG. 1. *Pyrochromatograms of (A) Vibrio cholerae, classical biotype, showing peaks α and β in complex 7; (B) Aeromonas sp., showing β but no α peak.*

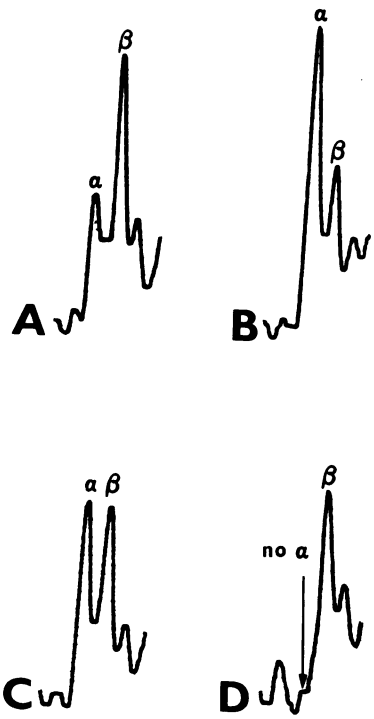

FIG. 2. *Complex 7 of the pyrochromatograms of (A) Vibrio cholerae, classical biotype, showing α peak smaller than β; (B) V. cholerae, El Tor biotype, showing α peak larger than β; (C) V. cholerae, intermediate biotype, showing similar-sized α and β peaks; (D) Aeromonas sp., showing β peak but no α.*

Category (iii). Peaks 7α and 7β were of more nearly equal size. These 26 strains were shown by conventional tests to belong to a group which might be designated *V. cholerae* (intermediate biotype). With some of them, the individual hemolysin, VP or polymyxin reactions were indeterminate. In others, the individual reactions were distinct, but, together, they did not conform to either the classical or the El Tor pattern: a commonly found combination (22 strains) was hemolysin-negative, VP-positive, polymyxin-resistant.

The reproducibility of the pyrochromatograms was good, and the difficulty in placing individual strains into the above three categories was no greater than with conventional tests. Somewhat arbitrarily, peak 7α was considered to be distinctly smaller than 7β if its height was less than half that of 7β, and distinctly larger than 7β if its height was more than twice that of 7β. Perhaps a more relevant comparison may have been made by comparing, with a digital integrator if one had been available, the areas under these two curves rather than the heights of the two peaks. But, even then, there would doubtless be border-line strains which might be difficult to assign to one of our three categories. However, similar difficulties were encountered with conventional tests in which, for example, border-line strains were not unequivocally VP-positive or VP-negative, nor were they clearly polymyxin-resistant or polymyxin-sensitive; and some strains, though giving clear results with individual tests, did not provide a recognized pattern.

The PGLC patterns remained similar when the original columns were replaced by a new pair of the same specification. Cholera-like vibrios were readily distinguishable from the nonvibrios by PGLC, so that this technique could be used as a screening test for the exclusion of organisms like *Aeromonas* with which vibrios are sometimes confused. Indeed, an organism that we received from Ghana as a cholera vibrio was readily shown by PGLC to be a nonvibrio and by conventional methods to be a strain of *Aeromonas* (straight rod, arginine-positive, with a negative cholera-red reaction).

Epidemiology. The strains identified as *V. cholerae* (classical biotype) included all six of the strains received from Calcutta that had been isolated from human sources in 1957 or 1960.

The strains identified as intermediate biotype, by both conventional methods (hemolysin-negative El Tor) and PGLC, included all five strains from Singapore (human sources in 1969), all seven of the cholera-like vibrios from Nigeria, and also three from human sources and two from water in Calcutta in 1966–67.

Thus, when used in association with conventional methods of identification, PGLC may be a most useful tool in a study of the epidemiology of cholera, and in indicating a possible common origin of outbreaks separated from each other in time or distance, or both.

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