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rRNA Gene Restriction Patterns of *Haemophilus influenzae* Biogroup Aegyptius Strains Associated with Brazilian Purpuric Fever

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The rRNA gene restriction patterns of 92 isolates of *Haemophilus influenzae* biogroup aegyptius, associated with conjunctivitis or Brazilian purpuric fever in the State of São Paulo, Brazil, were studied with 16 + 23S rRNA from *Escherichia coli* as a probe. All strains were classified into 15 patterns. Isolates from Brazilian purpuric fever cases were seen only in patterns 3 (most frequently) and 4 (rarely), whereas isolates from conjunctivitis were found in all 15 patterns. The study demonstrated that rRNA from *E. coli* can serve as a probe for molecular epidemiology.

In October 1984 in the small town of Promissão, State of São Paulo, Brazil, 10 children 0 to 10 years of age died after a purpura fulminans syndrome without any sign of meningitis. Almost all of these children had suffered from a purulent conjunctivitis 3 to 15 days before developing high fever, abdominal pain with vomiting, and purpura (2). This syndrome, called Brazilian purpuric fever (BPF) (2), was subsequently observed in 1985 in São José do Rio Preto (State of São Paulo) and in 1986 in Serrana, Fartura, Guatapará, and Presidente Prudente (State of São Paulo). A gram-negative bacterium reported as *Haemophilus aegyptius* was isolated from the blood of several patients with BPF (3).

At the same time, conjunctival cultures were obtained from patients with purulent conjunctivitis and normal controls in several towns of the State of São Paulo. Strains isolated from conjunctiva were reported as *H. aegyptius* (2). No epidemiological marker was available to differentiate BPF strains from other isolates with the characteristics of *H. aegyptius*.

A century ago, Koch (9) observed small bacilli associated with purulent conjunctivitis in Egypt. Weeks (18) isolated the organism, which was named *Haemophilus aegyptius* by Pittman and Davis (11). Kilian (8) found *H. aegyptius* to be phenotypically very close to *Haemophilus influenzae* biotype III. Several DNA hybridization studies showed *H. influenzae* and *H. aegyptius* to constitute a single DNA relatedness group (1, 6, 12). Casin et al. (6) proposed that *H. aegyptius* be considered as a subjective synonym of *H. influenzae*. Some minor genomic heterogeneity was observed in the *H. aegyptius-H. influenzae* genospecies, although no subspecies could be delineated (6). Although the specific epithet of *H. aegyptius* (Trevisan 1889) Pittman and Davis 1950 (14) was published earlier than the specific epithet of *H. influenzae* (Lehmann and Neumann 1896) Winslow, Broadhurst, Buchanan, Krumwiede, Rogers, and Smith 1917 (14), *H. influenzae* is the type species of the genus *Haemophilus* and cannot be replaced by another name unless action in that direction is taken by the Judicial Commission of the International Committee of Systematic Bacteriology. In this article, *H. influenzae* biogroup aegyptius will designate strains formerly labeled *H. aegyptius*.

It was recently shown (7) that, when total bacterial DNA is cleaved by a restriction enzyme and electrophoresed in agarose and the fragments are transferred to a filter and hybridized to ^{32}P-labeled *Escherichia coli* 16 + 23S rRNA, a rRNA gene restriction pattern can be visualized (after autoradiography) which is species specific. However, when some genomic heterogeneity is measurable by quantitative DNA hybridization, more than one rRNA gene restriction pattern can be observed (7).

With the purpose of differentiating BPF isolates from other *H. influenzae* biogroup aegyptius strains associated with conjunctivitis, we studied the rRNA gene restriction patterns of 92 isolates which included both BPF cases and non-case strains. The outcome of the study is the description of 15 rRNA gene restriction patterns, one of which was associated with most cases of BPF.

MATERIALS AND METHODS

Bacterial strains. Ninety-two *H. influenzae* biogroup aegyptius isolates were recovered from conjunctiva (72 isolates), blood (12 isolates), throat (5 isolates), cerebrospinal fluid mixed with blood (2 isolates), and skin scraping of

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petechial lesion (1 isolate). The patients were from Serrana (23 isolates); Ribeirão Preto (13 isolates); São José do Rio Preto (12 isolates); Promissão (8 isolates); Guariba (7 isolates); Lins (7 isolates); Presidente Prudente (4 isolates); Garça (3 isolates); Bastos, Piracicaba, Penha, and Restinga (2 isolates per town); and Fartura, Baurú, Guatapará, Dois Córregos, Barretos, Campinas, and Vista Alegre do Alto (1 isolate per town). The patients had either BPF or a purulent conjunctivitis, or they were asymptomatic.

All clinical isolates required hemin and NAD for growth, agglutinated human erythrocytes, hydrolyzed urea, and failed to produce indole, ornithine decarboxylase, and acid from D-xylose. The type strains of *H. influenzae* (NCTC 8143) and *H. aegyptius* (ATCC 11116) and strain Kilian 3, which was at the border of the *H. influenzae-H. aegyptius* genospecies (6), were included for comparison.

DNA preparation. Levinthal stock was prepared by adding 10% defibrinated horse blood to boiling brain heart infusion (Difco Laboratories, Detroit, Mich.). The medium was filtered through filter paper and sterile 0.22-μm nitrocellulose filters (Millipore Corp., Bedford, Mass.). Levinthal broth was prepared by mixing 1 volume of Levinthal stock and 3 volumes of Todd-Hewitt broth (Difco). For each strain, overnight shaken cultures at 37°C in 30 ml of Levinthal broth were centrifuged at 5,000 × *g*, and the pellet was suspended in 10 ml of a solution containing 0.1 M NaCl, 0.05 M EDTA, and Tris, pH 8.0.

The cells were lysed, and high-molecular-weight DNA was extracted and purified according to Brenner et al. (5).

Gel electrophoresis of endonuclease-cleaved DNA. Purified DNA samples (5 μg) were cleaved by restriction enzymes *Eco*RI and *Eco*RV (1 U/μg of DNA) according to the instructions of the manufacturer (Amersham International, Amersham, England). The digestion was done at 37°C for 16 h and stopped by heating at 65°C for 10 min. The separation of DNA fragments was done in 0.8% (wt/vol) agarose (type II, medium EEO; Sigma Chemical Co., St. Louis, Mo.) gel in Tris-acetate buffer (Tris-acetate, 0.04 M; EDTA, 0.002 M; pH 8.1) by horizontal electrophoresis (10).

After completion of electrophoresis, the DNA fragments in the gel were stained by ethidium bromide (1 μg/ml) and photographed under UV light.

Radioactive labeling of RNA and DNA. Ribosomal 16 + 23S RNA from *E. coli* (Boehringer GmbH, Mannheim, Federal Republic of Germany) was end labeled with [γ-^{32}P]ATP (Amersham) and a 5′ DNA terminus labeling kit with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), following the exchange reaction procedure and instructions provided by the manufacturer.

DNA from pBR322 was labeled with a nick translation kit and [α-^{32}P]dCTP (Amersham) following the instructions of the manufacturer.

Southern transfer and hybridization. The transfer of DNA fragments to Hybond-N nylon membrane (Amersham), prehybridization step, hybridization with labeled rRNA at 65°C for 17 h, washing, and autoradiography were described elsewhere (7).

DNA fragment size determination. DNA fragment size marker Raoul I (Appligene, Strasbourg, France) was electrophoresed side by side with cleaved DNA from *Haemophilus* strains for accurate determination of fragment sizes. The fragments which derive from pBR322 were visualized by hybridizing the nylon-bound fragments with labeled pBR322 DNA. These fragments have the following sizes (the 686-, 554-, 375-, and 234-base-pair [bp] bands were not used because of poor resolution, and the 48,502 and 10,620-bp bands do not reassociate with pBR322): 18,520, 14,980, 9,007, 7,378, 5,634, 4,360, 3,988, 3,609, 2,938, 2,319, 1,810, 1,416, 1,255, 1,050, 903, and 754 bp.

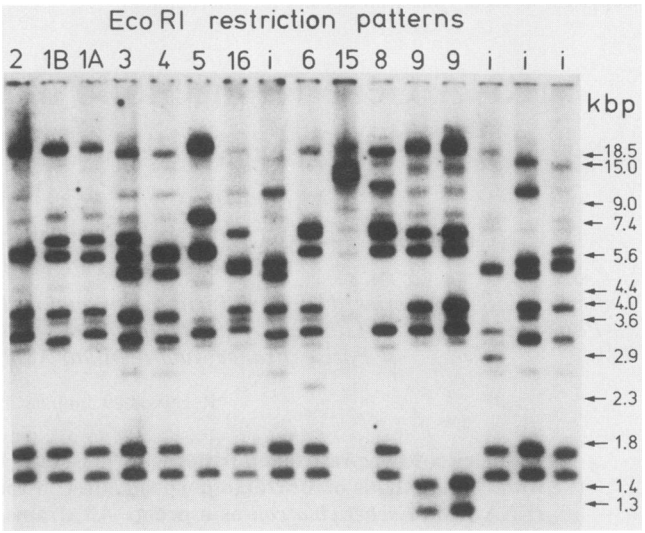

FIG. 1. Autoradiograph of a nylon membrane after transfer of *Eco*RI restriction fragments of *H. influenzae* biogroup aegyptius DNAs and hybridization with ^{32}P-labeled *E. coli* 16 + 23S rRNA. i, DNA from different strains of *H. influenzae* biogroup influenzae.

Fragment sizes were calculated from migration distances, using a personal TRS80 model 100 microcomputer and a BASIC program derived from that described by Schaffer and Sederoff (13). For fragment sizes of 754 to 9,007 bp, only the 14 fragments of Raoul I in that range were used as standard in the computation. This gave calculated sizes of standards within 1% of their actual sizes. For fragment sizes >9,007 bp, the six fragments of Raoul I ranging from 4,360 to 18,520 bp were used as standards in the computation. This gave calculated sizes of standards within 3 to 5% of their actual sizes. The use of the total set of standard fragments yielded poorer precision in the computations.

Earlier experiments used the rRNA gene restriction pattern of *Serratia fonticola* (seven fragments ranging from 5,400 to 14,500 bp) as a standard (7). However, the extrapolated fragment sizes were inaccurate when smaller than 5,400 bp. Side-by-side comparison of patterns was often needed to decide whether two bands were identical. This fragment size marker was abandoned when Raoul I was made available.

RESULTS

Figure 1 is a photograph of a typical autoradiogram showing rRNA gene restriction patterns. Strong bands and weaker bands are visible. With the exception of one strain which showed only three strong bands, each other strain studied showed six to eight strong bands and one to five weak bands which were not visible on all autoradiograms.

Figure 2 gives a normalized representation of patterns given by 92 clinical isolates of *H. influenzae* biogroup aegyptius and three reference strains. The fragment sizes indicated are average sizes. The standard deviation in the calculation of averages was 0.1 to 1.3% for sizes ranging from 1,000 to 7,500 bp and 2 to 4% for sizes >7,500 bp.

Fifteen major patterns were found among clinical strains. Pattern 1 was subdivided into 1A and 1B after side-by-side

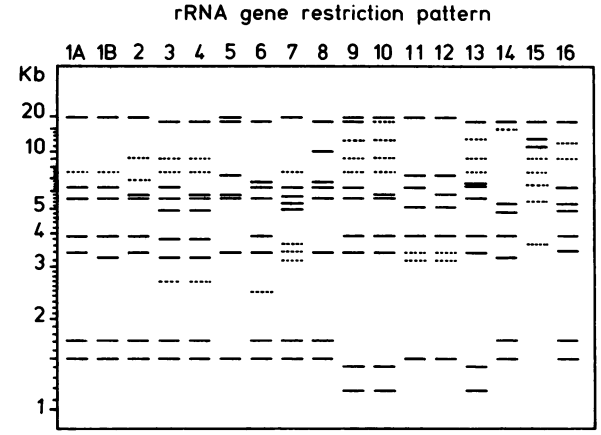

FIG. 2. Normalized graph showing migration patterns of rRNA gene restriction fragments after cleavage by *Eco*RI. Patterns 1A to 15 were given by *H. influenzae* biogroup aegyptius DNAs. Pattern 16 was given by DNA from the type strain of *H. influenzae* biogroup influenzae. The normalized migration values were obtained from mean fragment sizes by using one of the experimental formulas relating migration (M, in millimeters) to size (S, in base pairs): $M = 299{,}820/(S + 1{,}692) + 3$. kb, Kilobases.

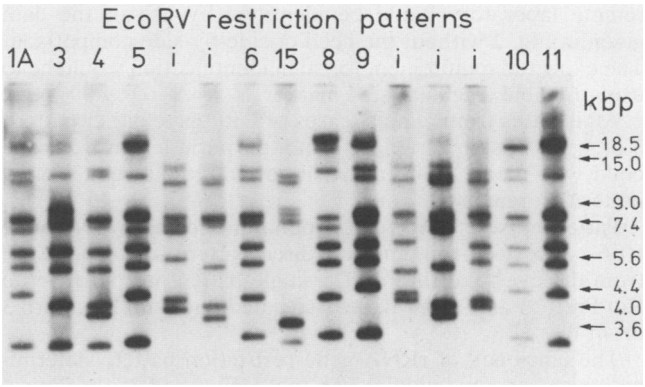

FIG. 3. Autoradiogram of a nylon membrane after transfer of *Eco*RV restriction fragments of *H. influenzae* biogroup aegyptius DNAs and hybridization with ^{32}P-labeled *E. coli* 16 + 23S rRNA. Pattern numbers are those obtained when *Eco*RI was used to classify the isolates (Fig. 2). i, DNA from different strains of *H. influenzae* biogroup influenzae.

comparison and precise size determination of the fifth strong band. Different numbers were not given to these patterns 1A and 1B because the difference might not be readily detected when these patterns are on different autoradiograms. The type strain of *H. aegyptius* gave pattern 1A, strain Kilian 3 gave pattern 15, and the type strain of *H. influenzae* gave pattern 16.

The distribution of rRNA gene restriction patterns of clinical isolates by source of isolation is given in Table 1. It is striking that all isolates from blood gave the same pattern 3. In contrast, all 15 patterns were seen among conjunctival isolates.

The geographical distribution of clinical isolates is as follows (number of isolates indicated when ≥4). The patterns found in the various towns were: Serrana, patterns 2 and 3 (22 isolates); Ribeirão Preto, patterns 1A, 1B, 2 (4 isolates), and 10 (5 isolates); São José do Rio Preto, patterns 2, 3 (5 isolates), 4 (5 isolates), and 10; Promissão, patterns 2, 3 (5 isolates), and 9; Guariba, pattern 5 (7 isolates); Lins, patterns 2, 6, 11, 12, 13, 14, and 15; Presidente Prudente, patterns 2, 3, and 10; Garça, pattern 1B; Bastos, patterns 1B and 3; Piracicaba, pattern 3; Penha, pattern 2; Restinga, patterns 1A and 7; Fartura, pattern 4; Guatapará, pattern 3; Dois Córregos, pattern 1B; Barretos, pattern 8; Campinas, pattern 2; Baurú, pattern 9; and Vista Allegre do Alto, pattern 2. Thus, pattern 3 was found in the towns of Serrana, São José do Rio Preto, Presidente Prudente, Bastos, Piracicaba, and Guatapará, whereas pattern 4 was limited to São José do Rio Preto and Fartura. Pattern 2 was found in nine towns, whereas pattern 5 was confined to a single town (Guariba).

An example of rRNA gene restriction patterns obtained by DNA cleavage with *Eco*RV endonuclease is shown in Fig. 3. Patterns 3 and 4, which differed by the position of only one band when *Eco*RI endonuclease was used (Fig. 2), are again different when *Eco*RV endonuclease is used. All DNAs were not submitted to cleavage by *Eco*RV since preliminary results did not yield additional information to that gained with *Eco*RI.

DISCUSSION

The determination of rRNA gene restriction patterns can be useful for the identification of homogeneous genomic species of bacteria (7). In some species, however, several patterns can be observed (7) and thus have epidemiological interest. The present work reports the use of rRNA as a probe in molecular epidemiology. Molecular epidemiology can also use plasmid restriction profiles (16) or randomly cloned DNA fragments to visualize patterns in Southern blots (17). The advantage of our method over the use of randomly cloned DNA fragments is that, because rRNA has been more conserved during evolution than the rest of the genome, a single probe (rRNA from *E. coli*) is applicable to the study of rRNA gene restriction fragments of any kind of bacteria (7). Plasmid restriction fingerprinting is a good epidemiological method only when a plasmid is present in the studied bacterium. Furthermore, only plasmids are compared, not the bacterial host.

This work also describes the first comprehensive typing system for *H. influenzae* biogroup aegyptius, since all strains are typable. The patterns which would be observed in a

TABLE 1. Sources of Brazilian *H. influenzae* biogroup aegyptius isolates with known rRNA gene restriction pattern

Clinical source	No. of strains with given rRNA gene restriction pattern															
	1A	1B	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Blood	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0
Skin	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Cerebrospinal fluid (with blood)	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Throat	1	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0
Conjunctiva	2	7	15	21	4	6	1	1	1	2	7	1	1	1	1	1

remote laboratory could be identified by use of the data given in Fig. 2 without the need of side-by-side comparison. The expected requirement for such identification would be to use a reliable fragment size marker.

All isolates from clinical cases of BPF gave either pattern 3 or 4 (4). A skin scraping from a purpuric lesion from one child who died (2) and all positive blood cultures (3) grew pattern 3 organisms.

Pattern 4 isolates were not found in Serrana, where most BPF cases occurred. In fact, three pattern 4 isolates were from the eyes and throat of a single patient in São José do Rio Preto. Fatal cases were associated with either pattern 3 or 4 (4).

The efficiency of rRNA gene restriction pattern determination as an epidemiological tool compared with clinical history, plasmid restriction fingerprinting, protein electrophoresis, and multilocus enzyme electrophoresis was demonstrated in a companion paper (4). A difference occurred in the case of RNA patterns 3 and 4, which were not separated by other methods. These patterns are likely to represent two different clones since differences in patterns obtained with *Eco*RI were confirmed by differences in patterns with *Eco*RV (the cleavage sites of these enzymes are different).

It would be interesting to determine the rRNA gene restriction patterns of *H. influenzae* biogroup aegyptius isolated in different countries and to see whether pattern 3 or 4 occurs.

The rRNA gene restriction patterns of Brazilian strains of *H. influenzae* biogroup influenzae are being studied in our laboratory. Preliminary results show a large variety of patterns different from those described here for biogroup aegyptius.

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ADDENDUM

Examples of use of rRNA in molecular epidemiology were given in a recent paper (15). One of the examples included 10 strains of *H. influenzae*.

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