Ribotyping, Biotyping and Capsular Typing of *Haemophilus influenzae* Strains Isolated from Patients in Campinas, Southeast Brazil

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Forty-five *Haemophilus influenzae* strains isolated from patients were characterized based on biochemical characteristics. Their capsular types were determined by polymerase chain reaction (PCR); they were compared, using two molecular methods [ribotyping with a specific DNA probe amplified from the 16S rDNA region from *H. influenzae* and through restriction fragment length polymorphism (RLFP) of an amplified 16S DNA region]. The strains were better discriminated by the ribotyping technique that used the 16S probe and by the combination of both techniques. Biotypes I and IV were the most common, followed by biotypes VI, VIII and III. Biotypes II and VII were not found. Most of the capsular samples were nontypable (89%), with capsular types a and b found in 2 and 9% of the samples, respectively. We concluded that there is a very close genetic identity among pathogenic and non-pathogenic strains.

Key-Words: *Haemophilus influenzae*, characterization, ribotyping, serotype, biotype.

Haemophilus influenzae, a commensal pleomorphic Gram-negative bacillus found in the human upper respiratory tract, has been associated with localized and invasive infections, such as bronchitis, otitis, pneumonia, meningitis, septicemia, and epiglottitis [1]. Systemic diseases are normally caused by *Haemophilus influenzae* serotype b (Hib) strains. The isolates obtained from infectious processes of the respiratory tract and from healthy individuals are usually unencapsulated and nontypable (NTHI) [2]. The current classification of this species is based on biotypes and capsular serotypes, which are subject to phenotypic variations and do not provide any clonal origin data [3]. Other typing methods, such as outer membrane protein analysis, lipopolysaccharide profiling, and multilocus enzyme electrophoresis, have been used to study the epidemiology and pathogenesis of *H. influenzae* infections [4]. Recently, *H. influenzae* strains have also been genomically characterized by determination of capsular types based on biomolecular techniques, including PCR-ribotyping [5], RAPD-PCR [6], PFGE [4], and PCR [7,8], all of which have revealed a great variety of patterns.

Forty-five *H. influenzae* isolates from different body fluids of patients attended at the Medical School Hospital of the Universidade Estadual de Campinas (UNICAMP), Brazil between 1997 and 1998 were studied, using a combination of two different ribotyping methods. First, RFLP was used to amplify and cut a specific 16S rDNA region of H. influenzae with restriction enzymes, and then a DNA probe of the 16S rDNA region was used to hybridize with the restricted genomic DNA, in order to assess strain variability.

The biotype of all strains was also studied by biochemical methods [1], and their capsular types were determined by PCR reaction using specific primers for each capsular type (a-f) [7]. All the results were compared to establish genetic identity among the different strains.

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Material and Methods

Bacterial Strains and Media

The 45 *H. influenzae* strains used in this study were isolated from body fluids collected from patients attended at the Medical School Hospital of the Campinas (São Paulo) State University, from 1997 to 1998 (Table 1). These strains were cultivated under agitation (150 rpm), at 37°C in Brain Heart Infusion (BHI) broth supplemented with haemin (10 µg/mL) and NAD (2 µg/mL) [9]. When necessary to grow the strains on solid medium, the BHI medium was supplemented with 1.5% agar. All strains were stored at –80°C in BHI medium containing 15% glycerol.

Strain Biotyping

The strain biotyping media described by Kilian were used [1]. The strains were biotyped based on ornithine decarboxylase, urease, indol production, and D-xylose fermentation.

Strain Capsular Typing

Capsule types (a-f) were determined using primer pairs for each capsular type. Primer pairs and the PCR reactions were as described by Falla et al. [7].

Genomic DNA Extraction

Genomic bacterial DNA was extracted as described by Ausubel et al. [10]. The extracted DNA was resuspended in TE buffer plus 10mg/mL of RNAse, and its integrity was determined using 0.7% agarose gels in TE buffer, as described by Sambrook et al. [11].

PCR Amplification of 16S *rDNA*

The sequences of primers for the PCR 16S *rDNA* amplification were obtained from the Gene bank sequence

accession number GI: 3551859 (*16SForward:* 5'-GCTGACGAGTGGCGGACGGG-3'; *16SReverse:* 5'-GCTCGTTGCGGGACTTAACC-3'); 200 ng of genomic DNA was submitted to PCR amplification in a final volume of 50 µL containing 10mM Tris-HCl (pH8.4); 50 mM KCl; 1.5 mM MgCl; 50 pmol of each primer; 0.2 mM of each deoxyribonucleoside triphosphate; 2.5 U of *Taq* DNA polymerase (Gibco BRL, Life Technologies). PCR reactions were run as follows: an initial denaturation (95°C, 5 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (61°C, 1 min), and extension (72°C, 2 min) with a final single extension (72°C, 7 min). The size (1,100bp) of the expected fragment was visualized by electrophoresis in submersed agarose gels (1.5%), using a 1kb DNA marker (Gibco BRL, Life Tech), as a standard.

RFLP of 16S rDNA with Enzymes *BamHI* and *HaeII*I

For each strain, 500 ng of the amplified 16S *rDNA* fragment (1,100 bp) was restricted either with *BamHI* or *HaeIII* enzymes, and the product separated using submersed agarose gel (1.5%) electrophoresis. Visualization of the obtained fragments was accomplished as described by Sambrook et al. [11].

Non Radioactive-Labeling of the 16S *rDNA* Probe

The 16S *rDNA* fragment obtained after PCR amplification was labeled following the specifications described in the BioPrime TM DNA Labeling System instructions (Gibco BRL, Life Technologies).

Southern Blotting

For each strain, 5µg of the genomic DNA was digested with 20U of *EcoRI* (Gibco BRL, Life Technologies) and submitted to electrophoresis in a submersed agarose gel (0.7%). The DNA was transferred to nylon membranes, as described by Sambrook et al. [11]. The 16S *rDNA* hibridization and membrane staining conditions followed the protocols described in the BluGene Nonradioactiove Nucleic Acid Detection System (Gibco BRL, Life Technologies).

Data Analysis

The data were analyzed with the POPGEN 32 package [12] and program UPGMA [13]. The presence of a given band was coded as 1 and its absence as 0 in a data matrix. Dissimilarity dendrograms were constructed for each case.

Results

The forty-five *Haemophilus influenzae* strains were isolated from November 1997 to June 1998 from patients attended at the Medical School Hospital of The Campinas State University (UNICAMP) and were classified either according to their site of isolation or the type of pathology with which they were associated (Table 1). Among these strains, 13% (n = 6) were isolated from upper-respiratory-tract infections, 60% (n = 27) from lower-respiratory-tract infections, 11% (n = 5) from cerebral spinal fluid, 7% (n = 3) from blood culture, 7% (n = 3) from eye infections and 2% (n = 1) from sinovial fluid (Table 1).

Biotyping demonstrated that 51% of the strains belonged to biotype I, 2% to biotype III, 31% to biotype IV, 2% to biotype V, 7% to biotype VI, and 7% to biotype VIII. Biotypes II and VII were not found (Table 1).

Biotype and strain isolation origin were characteristically associated. Although the strains isolated from cystic fibrosis patients were mainly biotypes IV (47%) and I (29.5%), biotypes VI and VIII were also isolated in 6% and 17.5% of the cases, respectively. Invasive strains expressed biotype I (three strains isolated in meningitis cases and one strain isolated from blood culture), biotype IV (two strains isolated in meningitis cases and one strain from blood culture), and biotype V (one strain isolated from blood culture).

Capsular type characterization showed that most strains (89%) were capsulated and nontypable, while only 2 and 9% of the strains had capsule types a and b, respectively. Type-a strain was isolated from a pneumonia case, while type-b strains were isolated from blood culture (two strains) and cerebral spinal fluid (two strains).

Amplification of 16S *r*DNA produced a DNA fragment of approximately 1,100bp. Digestion of this fragment with *Bam*HI produced a restriction pattern with two types of fragments, one with a non-digested fragment of approximately 1,100bp and another with fragments of approximately 880 and 160bp. Digestion with *Hae*III also produced two types of restriction patterns: one of approximately 785 and 235bp and another with approximately 480, 180, and 143bp fragments (fragments corresponding to differences in size not observable in the gels are not shown). Together, these data showed a high genetic similarity between strains, indicating only two (A-B) strain clusters (Figure 1). Cluster A comprised nearly all strains (93.3%) and cluster B had only three strains. This analysis grouped most of the strains very close together, irrespective of biotype and capsular type, and showed very low variability between strains.

The construction of a molecular probe from a 1,100bp fragment of the 16S*rDNA* gene for hybridization with the restricted genomic DNA allowed identification of DNA fragments with molecular weights ranging from 195 to 19,212bp (data not shown). It resulted in eight (1-8) main strain clusters; however, they presented low dissimilarity between strains (0-25%, Figure 2).

Clusters 1 and 4 contained five different strain clones, cluster 2 contained four clones, cluster 3 contained seven clones, clusters 5-8 contained either one or two strains each. Clusters 1 and 2, the ones with the lowest dissimilarity, contained all biotype VIII strains, most biotype IV strains (71.4%), and only eight (33.3%) biotype I strains, indicating a possible separation of strains with biotype IV.

Most strains (94.4%) isolated from cystic fibrosis patients were grouped in clusters 1-3 with a maximum dissimilarity of approximately 15%. However, the genomic structures of the most invasive strains, such as strain 40 (cerebral spinal fluid,

Table 1. Isolation sites or body fluids from which the *Haemophilus influenzae* strains were isolated (NT = nontypable *H. influenzae*, a = serotype a, b = serotype b).

Strains	Strain origin	Biotype	Serotype
1	Cystic Fibrosis	I	NT
2	Cystic Fibrosis	I	NT
3	Cystic Fibrosis	VIII	NT
5	Cystic Fibrosis	IV	NT
6	Cystic Fibrosis	IV	NT
7	Cystic Fibrosis	IV	NT
8	Cystic Fibrosis	I	NT
9	Cystic Fibrosis	IV	NT
10	Cystic Fibrosis	IV	NT
11	Cystic Fibrosis	IV	NT
12	Cystic Fibrosis	IV	NT
13	Cystic Fibrosis	I	NT
14	Cystic Fibrosis	I	NT
15	Cystic Fibrosis	VI	NT
16	Cystic Fibrosis	IV	NT
17	Cystic Fibrosis	VIII	NT
18	Cystic Fibrosis	VIII	NT
19	Cystic Fibrosis	IV	NT
20	Bronchial Infection	VI	NT
21	Amigdalytis	III	NT
22	Amigdalytis	I	NT
23	Bronchial-Alveolar Secretion	I	NT
24	Bronchial-Alveolar Secretion	I	NT
26	Ocular Secretion	I	NT
27	Ocular Secretion	IV	NT
28	Ocular Secretion	I	NT
29	Sinus disease	I	NT
30	Sinovial Liquid	VI	NT
31	Sinusitis	I	NT
32	Bronchial Secretion	I	NT
33	Bronchial Secretion	IV	NT
34	Bronchial Secretion	I	NT
35	Sputum	I	NT
36	Pneumonia case	I	NT
37	Pneumonia case	I	NT
38	Pneumonia case	I	A
39	LCR – Meningitis	IV	NT
40	LCR – Meningitis	I	NT
41	LCR – Meningitis	I	NT
42	LCR – Meningitis	I	B
43	LCR – Meningitis	IV	B
44	Pleural Liquid	I	NT
45	Hemoculture – Meningitis	V	B
46	Hemoculture – Meningitis	I	B
47	Hemoculture – Meningitis	IV	NT

Figure 1. Dendrogram of dissimilarity obtained for *Haemophilus influenzae* strains using the RFLP of amplified 16S *r*DNA (bars = percentage of dissimilarity).

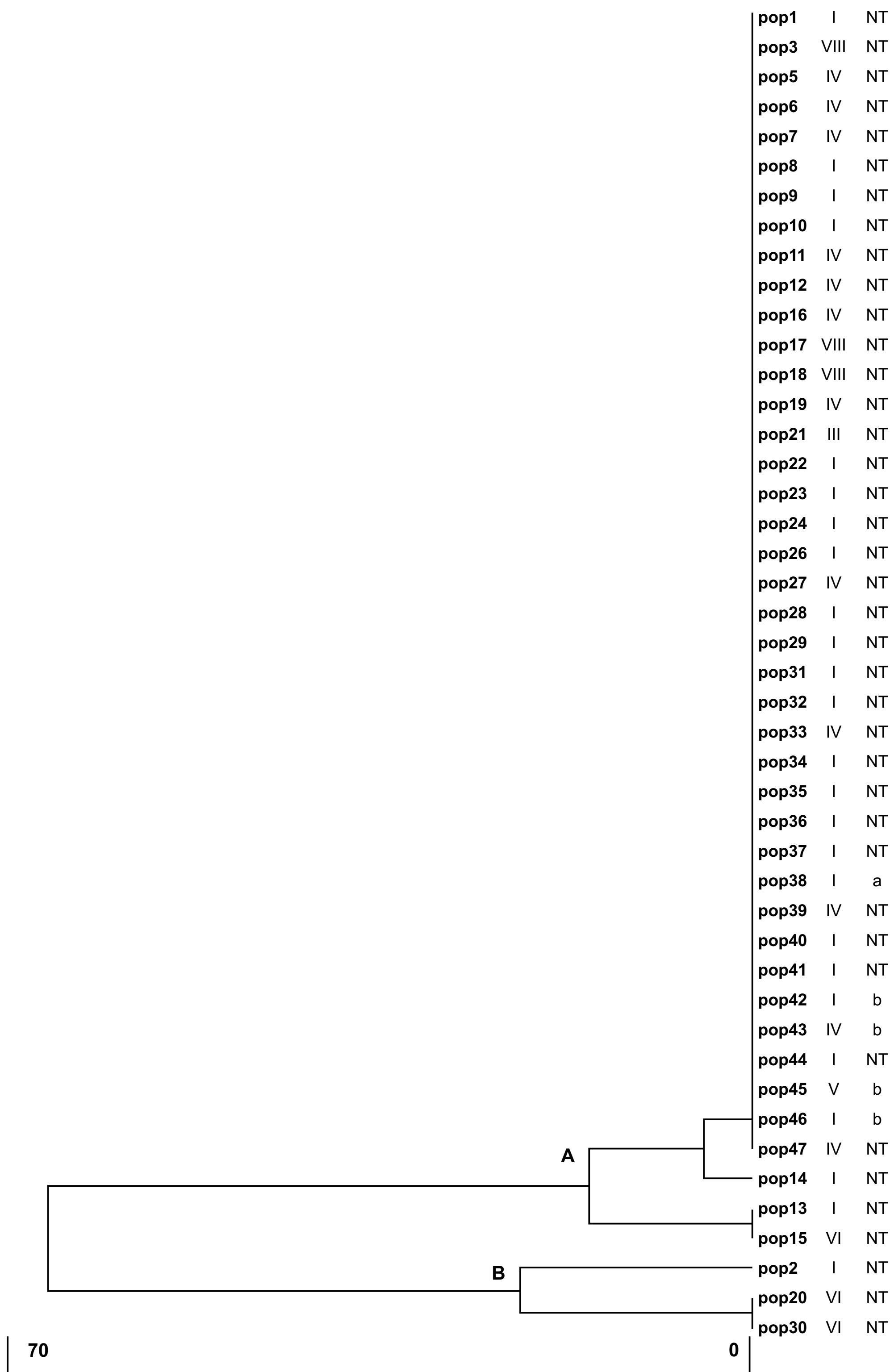


Figure 2. Dendrogram of dissimilarity obtained for *Haemophilus influenzae* strains using the amplified 16S *r*DNA region as a molecular probe (bars = percentage of dissimilarity).

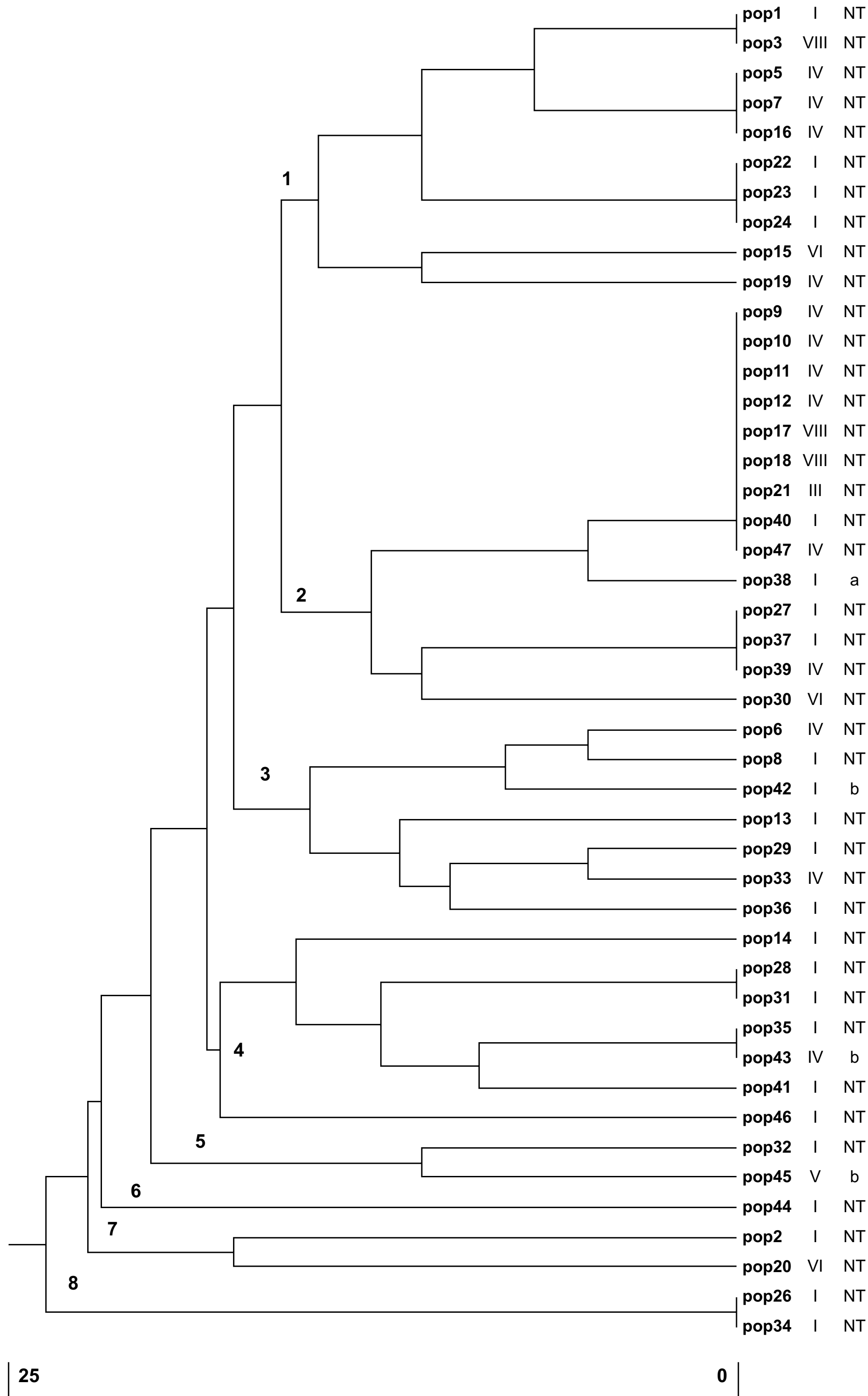
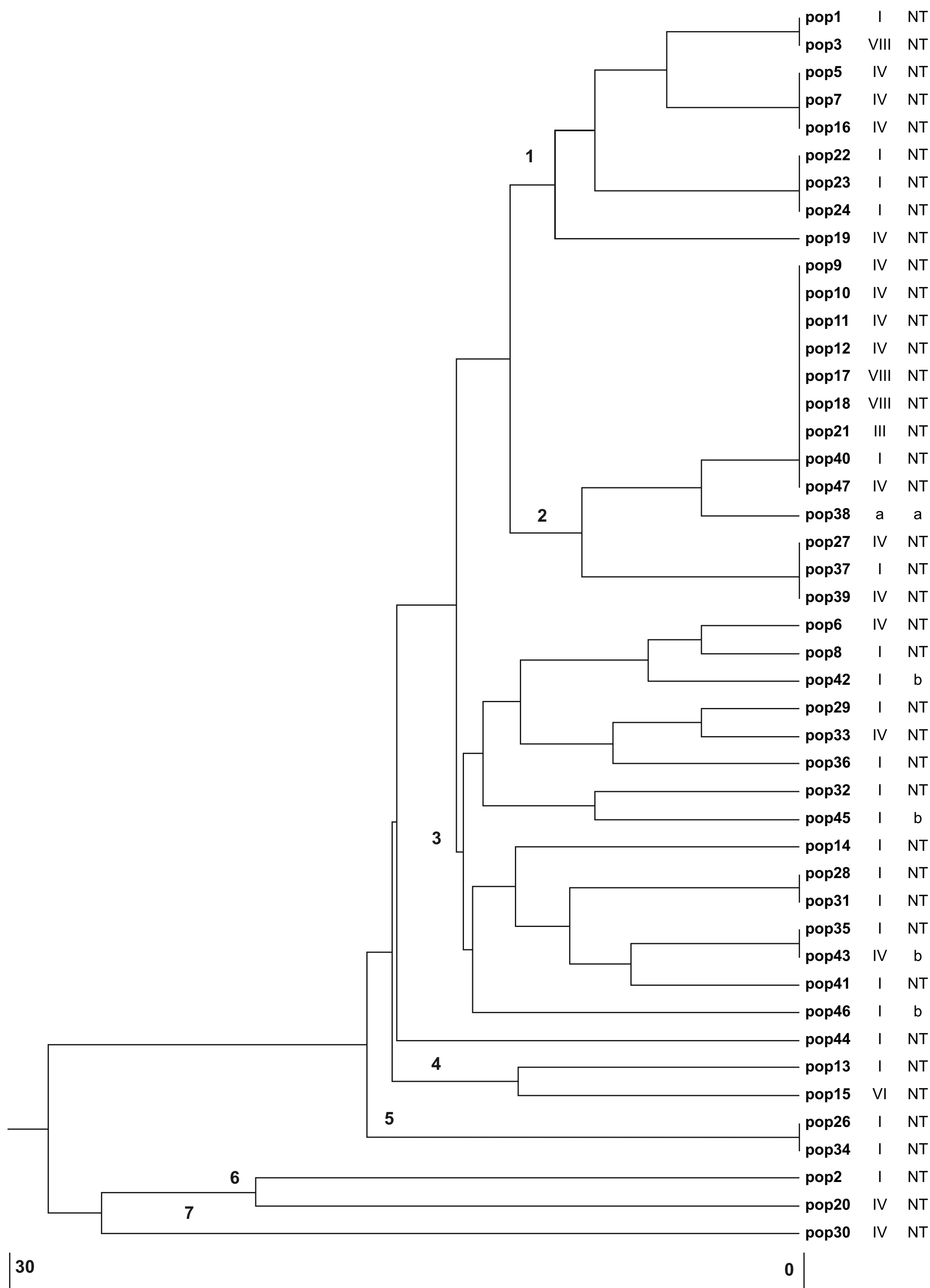


Figure 3. Dendrogram of dissimilarity obtained for *Haemophilus influenzae* strains using 16S *r*DNA *r*DNA region and the amplified 16S *r*DNA region as a molecular probe (bars = percentage of dissimilarity).



biotype I, nontypable) and strain 47 (blood culture, biotype IV, nontypable) were either identical (strains 9-12, 17, and 18, for example) or very similar (strains 1 and 3, for example) to those strains isolated from cystic fibrosis patients. Likewise, strain 35 (sputum, biotype I and nontypable) and strain 43 (cerebral spinal fluid, biotype IV, capsular type b) (cluster 4) were identical strains by this approach. In addition, strains with theoretically the same pathogenic capacity were found in distinct clusters (strains 39 and 42, in clusters 2 and 3, respectively).

The combination of both techniques allowed us to construct a dendrogram with seven clusters (Figure 3), which was very similar to that constructed using the previous technique based on strain biotype and origin; but some strains, such as 15, 20, and 30 (all biotype VI) were reclustered in low genetic similarity clusters 4, 6, and 7, respectively.

Discussion

We used two technical variants of the same molecular assay (RFLP of 16S *r*DNA cut with two different restriction enzymes and RFLP of genomic DNA with 16S *r*DNA fragments as detected by a molecular 16S probe) and their combination to examine clonal variability of clinical isolates of *H. influenzae*. Complementary biological characteristics, biotype, capsular type, and isolation site were determined for strain identification and characterization.

As *H. influenzae* is an important cause of human disease worldwide and serotype b (Hib) capsulated strains cause invasive infections, such as meningitis, septicemia, and septic arthritis, particularly in infants, many classification methodologies have been tried to better determine their virulence [4-8].

Haemophilus influenza is traditionally characterized by determination of biotype and capsular serotypes [14,15]. These methods are subject to phenotypic variations and do not provide information on the strain's clonal origin [16]. Biochemical discriminatory methods, such as outer membrane protein analysis, lipopolysaccharide profiling, multilocus enzyme electrophoresis, and several biomolecular techniques, including analysis of DNA restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), and amplification and enzyme restriction of the *rDNA* gene, have been used in epidemiological and pathogenesis studies of *H. influenzae*. Amplification and enzyme restriction of the *rDNA* gene uses the ribosomal region as a typing and characterization target. RFLP analysis, using *r*RNA (cloned *r*DNA) as a probe, called ribotyping, has also been employed in the differentiation of several bacterial species isolates (Smith-Vaughn et al., 1995). Generally, genetic studies of *H. influenzae* use PCR-ribotyping with amplification of the 16s RNA region, followed by enzyme restriction [17].

We found that only five strains (one type a and four type b) had a typable capsule. Epidemiological studies [17-19] have shown a high incidence of type b capsule among strains. Our results revealed more nontypable capsular strains, with good indication of isolation site. Most strains were non-invasive and isolated from cystic fibrosis and other respiratory tract clinical cases.

Most of the strains were biotypes I, IV, VI, VIII, III, and V, in decreasing order of frequency. These data are somewhat similar to what was found by Foxwell et al. [3] and Saito et al. [22], who reported that biotypes I, II, III, and IV were most frequent.

In addition, strain 45 expresses capsular type b and is biotype V. Till now, it was believed that invasive strains expressing this capsular type would belong only to biotypes I, II, or III [1].

Strains expressing capsular type b are considered to be the main ones responsible for meningitis in children up to five years of age [18-22]. We have also isolated nontypable *H. influenzae* strains (NTHi) in meningitis (strains 39; 40 and 41) and septicemia (strain 47) cases. The current practice of vaccination against type b *H. influenzae* during early childhood in Brazil may be selecting virulent strains that otherwise would have no epidemiological importance. A naturally competent bacteria capable of acquiring exogenous DNA from other strains is another factor to be considered in the exchange of virulence genes between strains of *H. influenza* (reviewed by Marrs et al. [23]), as it would favor the appearance of new pathogenic strains. In addition, differently from the results obtained by Saito et al. [19], we did not find strains expressing capsular types c, d, and f.

Among the techniques that we used to assess the clonal structure of this bacterial population, amplification of rDNA, followed by enzyme restriction showed a low discriminatory power among strains, since it yielded only two clusters. The discriminatory power increased when either 16S *r*DNA fragment was used as a molecular probe against total genomic DNA or with a combination of both analyses. We suggest that the use of this approach would give better results in epidemiological studies.

Independent of the method used to differentiate strains, non-invasive strains isolated from respiratory tract infections have a very close genetic identity with invasive pathogenic strains, which suggests that these two subgroups of bacteria have a common ancestor.

The similarity of the profiles of several strains found by these ribotyping techniques led to the conclusion that pathogenic and non-pathogenic *H. influenzae* strains could have similar or identical pathogenic mechanism genes. Different conditions would permit the expression of specific genes in specific microenvironments, thus regulating the expression of virulence factors according to host conditions. Again, exchange of DNA by lateral gene transfer and recombination, masking the prevalence of specific clones in the population of bacteria should also be considered, since *H. influenzae* is a naturally competent species, as previously reported [23,24]. Nevertheless, ribotyping allowed analysis of the genetic similarity of strains with phenotypic differences associated with the types of disease and the isolation sites.

In conclusion, we found that: (i) characterization of *H. influenzae* strains using only the classic ribotyping technique is not enough to show the real variability between strains, (ii) a combination of techniques using different ribotyping techniques affords a higher discriminatory power, (iii) most of the strains were biotype I, (iv) most of the strains had an nontypable capsule, (v) one biotype V serotype b strain was first found responsible for meningitis in Brazil, (vi) nontypable strains were isolated from meningitis and septicemia cases, (vii) no strains expressing capsular types c, d, and f were detected, (viii) non-invasive strains isolated in respiratory tract infections have a very close genetic identity with invasive pathogenic strains, which indicates that pathogenic and non-pathogenic *H. influenzae* strains may have similar or identical pathogenic mechanism genes. Different conditions lead to the expression of specific genes in specific microenvironments, thus regulating the expression of virulence factors according to host conditions.

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