

MOLECULAR EPIDEMIOLOGY

Subtype distribution of *Haemophilus influenzae* isolates from North India

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A total of 120 *Haemophilus influenzae* isolates from blood, cerebrospinal fluid, sputum and throat swabs of patients and carriers in North India was characterised by biotyping, ribotyping and random amplification of polymorphic DNA (RAPD)-PCR. Of these, 77 isolates (64%) were serotype b; the other 43 (36%) were non-typable. Biotype I was the most predominant among the typable strains and biotype II among the non-typable strains. Ribotyping with restriction endonucleases *HaeIII* and *EcoRI* differentiated the isolates into three and six ribotypes, respectively. However, RAPD fingerprints generated by the application of arbitrary primers AP1 and AP2 provided a higher level of discrimination. RAPD typing revealed distinct polymorphism among the serologically typable isolates. This study is the first report that stratifies the subtypes of *H. influenzae* strains from India by molecular techniques.

Introduction

Haemophilus influenzae, although a common commensal of the upper respiratory tract of healthy individuals [1], is an important pathogen. The species is divided into six capsular types on the basis of production of a polysaccharide capsule [2] of which type b is a frequent cause of meningitis and other invasive disorders in children [3]. The non-capsulate strains also cause a wide spectrum of clinical infections including chronic bronchitis, pneumonia and bacteraemia, and are found in 75% of healthy individuals [4].

In India, 96% of all haemophilus infections are caused by *H. influenzae* serotype b (Hib) [5]. Since the mid-1950s, Hib has been the most common cause of pyogenic meningitis in children in India. The burden of invasive Hib is substantial, with the incidence of Hib meningitis estimated to be 50–60 cases/100 000 children <5 years of age [6]. A study from Delhi has also reported Hib to be a common cause of pneumonia in 19% of cases in children [7]. The outcome has not changed significantly in the past decade despite the introduction of potent antibiotics.

The large and genetically diverse pool of *H. influenzae*

strains circulating in the community has been documented previously [8]. Therefore, the availability of high resolution typing assays is a prerequisite for the study of *H. influenzae* epidemiology. The investigation and comparison of outbreaks of infection have been hampered by the lack of standardised, highly discriminatory methods for characterising the strains. The use of several molecular typing techniques for the effective detection of outbreaks of infection as well as identification of new and infectious clones of *H. influenzae* has been reported in recent years [9, 10]. Among these, restriction fragment length polymorphism analysis with ribosomal RNA (rRNA) as the probe [11–13] has been used widely to differentiate isolates into ribotypes. Intra-specific genetic variation can also be detected by randomly amplified polymorphic DNA (RAPD) analysis. In RAPD, the amplicons, when arrayed by electrophoresis, yield fingerprints which differ depending on the relatedness of the genomic templates [14].

The aim of this study was to characterise the *H. influenzae* isolates from patients and carriers by biotyping, ribotyping and RAPD fingerprinting and thus to analyse the subtype distribution of isolates from India.

Materials and methods

Study population

Seventy-seven *H. influenzae* type b isolates from cerebrospinal fluid (CSF) and blood samples of infants

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(aged <1 year) suffering from meningitis and bacteraemia, respectively, attending clinics at the Postgraduate Institute of Medical Education and Research, Chandigarh, India, were examined. Forty-three nontypable isolates were cultured from throat swabs of children (aged 5–15 years) and adults (aged 23–40 years) with informed consent and from the sputum of patients (aged 25–35 years) suffering from chronic bronchitis. Patients with a history of antibiotic therapy and those currently receiving antibiotics were excluded. All the samples were collected between March 1997 and July 2000.

Bacterial isolates

The samples collected from the patients and carriers were cultured on 7% chocolate agar in an atmosphere containing CO₂ 5%. *H. influenzae* isolates were identified by their typical colony morphology and growth requirements for NAD and haemin. Serotyping was performed by slide agglutination tests with antisera to the capsular antigens a–f (Difco). Biotypes were assigned by testing *H. influenzae* isolates for the ability to produce urease, indole and ornithine decarboxylase by the method of Kilian [15]. The isolates were stored at –70°C in Brain Heart Infusion Broth (Difco) supplemented with glycerol 5% v/v.

Preparation of genomic DNA

Genomic DNA was extracted by a modification of the method described by Pitcher *et al.* [16]. Briefly, the bacterial colonies from overnight cultures were suspended in 150 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and lysed by adding 450 µl of solution containing 5 M guanidium thiocyanate, 100 mM EDTA and sarkosyl 0.5% v/v. After incubation at 4°C for 10 min, 7.5 M cold ammonium acetate was added. The DNA was extracted with phenol-chloroform, precipitated with ethanol and finally dissolved in TE. DNA preparations from different samples were quantified spectrophotometrically and stored at –20°C for further use.

Ribotyping

Genomic DNA (10 µg) digested with restriction endonucleases *Hae*III and *Eco*RI (Roche, Germany) was separated in agarose 0.8% gel. Southern hybridisation was performed by standard procedures [17]. The probe used was a synthetic oligonucleotide (5′-AAG AGT TTG ATC CTG GCT CAG-3′) from bacterial 16S rRNA (Biobasics, Canada) and was prepared by end-labelling with $\gamma^{32}\text{P}$ -ATP (BRIT, Hyderabad, India). The membranes were hybridised with the probe, washed and autoradiographed by exposure to X-ray film by the method of Bruce and Jordens [9].

RAPD fingerprinting

RAPD analysis was performed with primers (Genset, Singapore) AP1 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) and AP2 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) which were arbitrarily chosen from enterobacterial repetitive intergenic consensus sequences. PCR amplification was performed in a 25-µl volume mixture containing 100 ng of template DNA, 200 µM dNTPs, 200 ng of primer and 1 unit of *Taq* polymerase (Roche, Germany). The reaction mixtures were overlaid with a drop of sterile mineral oil (Sigma) in each PCR tube to avoid evaporation during cycling. A negative control containing all the ingredients except template DNA was run in parallel to each PCR experiment. After an initial denaturation for 5 min at 94°C, the PCR was performed for 35 cycles in an automated thermal cycler (Perkin-Elmer Cetus, USA) programmed for denaturation at 94°C for 1 min, annealing at 25°C for 1 min and extension at 72°C for 4 min. The amplification reactions were repeated three times to check the reproducibility of the RAPD-PCR banding profiles. The final extension time was 10 min at 72°C. The amplification products (12 µl) were electrophoresed in agarose 1% gels (FMC Bioproducts, USA) in Tris-borate-EDTA buffer containing ethidium bromide 0.5 µg/ml. These gels were visualised in a UV transilluminator (Fotodyne, USA) and photographed. Appropriate DNA mol. wt markers were used for sizing the bands in the gel. Finally, RAPD-PCR bands were interpreted by visual analysis of the polaroid pictures.

Discriminatory power of the typing methods

The discriminatory ability of each typing method shown in Table 2 was determined by calculating the numerical discrimination index (DI) by the method of Hunter and Gaston [18]. A DI of 1.0 indicates that the typing method is able to distinguish each strain from the test population. Conversely, a DI of 0 indicates that all the strains of the test population are indistinguishable.

Results

Genetic polymorphism of *H. influenzae* strains prevalent in India was studied by biotyping, ribotyping and RAPD analysis and compared for epidemiological purposes. The results of these typing techniques are summarised in Table 1. A total of 305 cerebrospinal fluid (CSF) and 41 blood samples from infants (aged <1 year) suffering from meningitis and bacteraemia, respectively, was screened; 73 *H. influenzae* serotype b isolates were obtained from CSF and 4 from blood. The throat swabs of 40 healthy children (aged 5–15 years) and 23 adults (aged 23–40 years), and the sputum samples of 25 patients (aged 25–35 years) suffering from chronic bronchitis were also screened; 25, 10 and

Table 1. Subtype distribution of *H. influenzae* isolates from North India

<i>H. influenzae</i> isolates	Ribotype with										RAPD-fingerprint with																												
	Biotype					<i>HaeIII</i>					<i>EcoRI</i>					AP1					AP2																		
	I	II	III	IV	V	A	B	C	A	B	C	D	E	F	a	b	c	d	e	f	g	h	i	j	k	l	m												
Type b	31	24	8	9	5	51	17	9	21	8	7	6	28	7	10	5	7	11	10	2	16	9	3	4	9	8	7	10	8	11	6	8	8	10	10	8	17	8	
Non-typable	11	17	12			22	7	14	7			15	21								43																		

8 non-typable isolates were obtained from the respective groups. Seventy-seven strains (64%) were serotype b and 43 (36%) were non-typable. A few type b strains that were isolated from the throat swabs of a healthy carrier population were not included in the study. Other capsulate serotypes of *H. influenzae*, i.e., a, c, d, e and f, were not observed.

Biotyping differentiated the set of isolates into six biotypes; biotypes VI and VIII were not found (Table 1). Biotypes I, II and III were common among both the serotype b and non-typable isolates. Biotype I was predominant (40%) among the typable strains in the study population, although most of the non-typable isolates (39%) were of biotype II. Biotypes IV and V were found only in the typable isolates while biotype VII was associated only with the non-typable isolates.

Ribotyping with *HaeIII* restriction endonuclease discriminated the *H. influenzae* isolates into three ribotypes, each characterised by the presence of four or five fragments of differing size ranging from 0.5 kb to 4.4 kb (Fig. 1a). Ribotype A was the most prevalent among both the typable (66%) and non-typable (51%) isolates (Table 1). Ribotype analysis on digestion with *EcoRI* revealed six distinct banding patterns, with band size ranging from 0.5 to 10 kb (Fig. 1b, Table 1) of which ribotype E was the most common among both serotype b (36%) and non-typable (49%) isolates. However, *EcoRI* restriction allowed minor differentiation among the non-typable isolates.

Intra-serotypic variation was also evident by RAPD fingerprinting. The *H. influenzae* isolates exhibited marked heterogeneity with each of the arbitrary primers AP1 and AP2. Arrays of fragments ranging from 0.2 kb to 1.5 kb in size were observed. All the major and minor bands that were reproducible by repeated experiments were scored. The serotype b isolates showed 10 distinct banding patterns with primer AP1 (Fig. 2a), although it did not allow any differentiation among the non-typable isolates. RAPD type g was the most common among the serotype b isolates (21%) and was shown by all the non-typable isolates (Table 1). Primer AP2 provided the highest level of discrimination with a DI value of 0.925 (Table 2) among the isolates where variation in both number and size of bands could be observed in the fingerprints (Fig. 2b). Each of the RAPD patterns of the non-typable isolates was distinct from those of the type b isolates. AP2 could also differentiate the non-typable isolates that showed similar profiles with AP1. Therefore, AP2 was found to be more discriminatory than primer AP1 (Table 2).

Among the typable *H. influenzae* isolates from blood, there was complete concordance between ribotyping with *EcoRI* and RAPD fingerprinting with both the primers. Three of the four isolates from blood were of ribotype A with *EcoRI* and RAPD type a with AP1 and

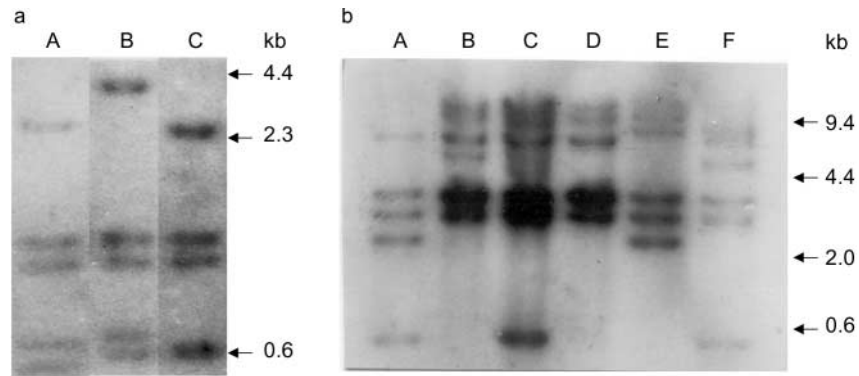


Fig. 1. Genomic ribotyping pattern of different subtypes of *H. influenzae* isolates following restriction enzyme digestion with (a) *HaeIII*, (b) *EcoRI*. Numbers indicate the molecular size corresponding to the marker (λ DNA, digested with *HindIII*).

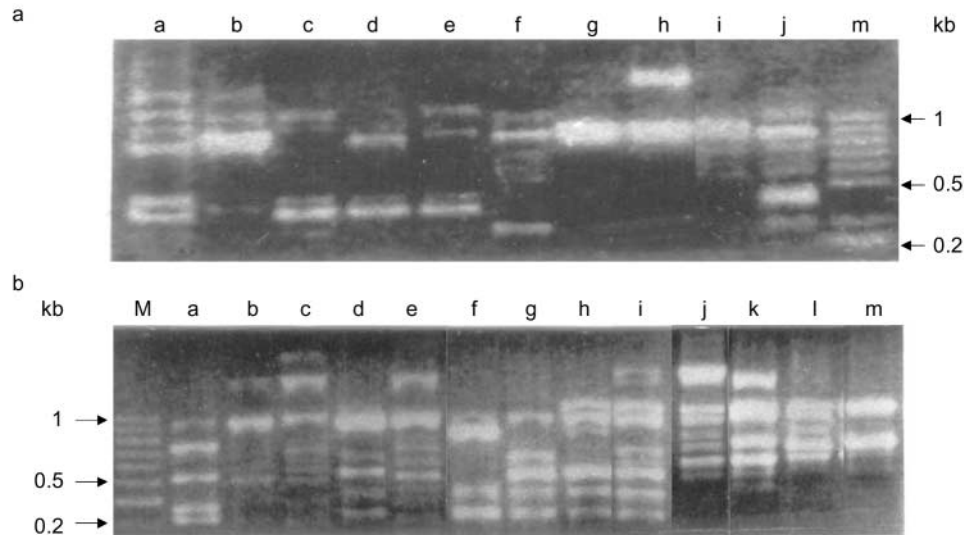


Fig. 2. RAPD-PCR amplification pattern of different subtypes of *H. influenzae* isolates with (a) AP1 primer, (b) AP2 primer. M, DNA molecular size marker.

Table 2. Discrimination indices [18] for the typing methods for *H. influenzae*

Typing method	Number of types	Discrimination index (DI)
Biotyping	6	0.731
Ribotyping with <i>HaeIII</i>	3	0.558
Ribotyping with <i>EcoRI</i>	6	0.743
RAPD fingerprinting with AP1	10	0.729
RAPD fingerprinting with AP2	13	0.925

AP2 whereas one isolate was of ribotype D and RAPD type d, respectively. All these four isolates belonged to ribotype A on digestion with *HaeIII*. Six of the isolates from the CSF samples belonged to ribotype A with the enzymes (*EcoRI* and *HaeIII*) and RAPD type a with both the primers and hence gave concordant profiles with all four genotyping methods.

Among the non-typable isolates, all eight isolates from the sputum samples of patients with chronic bronchitis showed complete concordance with *HaeIII* ribotyping

and RAPD analysis with both the primer sets. These samples showed ribotype A with *HaeIII* and RAPD type g and k with primers AP1 and AP2, respectively. *EcoRI* digestion further differentiated five of these isolates into ribotype A and three into ribotype E. However, the non-typable isolates from the throat swabs of the healthy carrier population demonstrated discordant results and were randomly distributed over the various subtypes.

Discussion

H. influenzae causes a wide spectrum of conditions that range from asymptomatic colonisation of the upper respiratory tract to serious infections such as meningitis. *H. influenzae* type b is a leading cause of bacterial meningitis and other invasive infections in childhood world-wide. In India, the annual incidence of Hib meningitis has been quite high and results in severe morbidity and mortality in children <5 years of age [5, 6]. In the present investigation also, 24% (73 of a

total 305) of the CSF and 10% (4 of 41) of the total blood samples screened were found to be positive for *H. influenzae* serotype b, which indicates a high prevalence of Hib-associated meningitis. Despite early diagnosis and appropriate treatment, Hib infection has been difficult to control and continues to pose an extensive health burden. Hence, there is an urgent need to study the epidemiology of *H. influenzae* in this community.

The ability to detect more subtle variations has increased substantially with the development of highly sensitive molecular techniques [9, 10]. Ribotyping [19] and RAPD-PCR [20, 21] have been used for detecting polymorphism within medically important organisms. However, the value of any typing procedure depends on the discriminatory power of the particular technique being applied.

Subtyping of *H. influenzae* by protein analysis has been done with strains from North America, Europe and UK [22, 23]. Molecular studies have also been done with these strains [24, 25] and also with Australian [26] and Japanese strains [27]. However, there has been a dearth of data on molecular analysis of strains from India. Hence we have, for the first time, subtyped *H. influenzae* isolates from India by genotypic methods.

In the USA, >95% of invasive *H. influenzae* are of serotype b and biotype I [28, 29]. In Western Europe, only biotypes I and II are prevalent in most countries although biotypes III, IV and V have also been detected [30]. In Japan, 73% of Hib strains belong to biotype I, 20% to biotype II and the remainder to biotype III. Serotypes a and c (found occasionally) are associated with biotypes I and I or IV, respectively, while the serologically non-typable isolates are distributed among all the eight biotypes, predominantly biotypes II and III [31]. In developing countries, 70–80% of *H. influenzae* strains are of serotype b. In a study from Pakistan, 64% of the samples screened were of serotype b and 98% of them belonged to biotype II [32]. However, in the present study, biotype I was the most predominant among the Hib strains, followed by biotype II among the serologically non-typable isolates. Interestingly, biotypes VI and VIII were not found in the population.

Several studies have used molecular strategies for typing *H. influenzae*. Ribotyping and RAPD analysis of strains from the UK revealed considerable heterogeneity among the non-typable isolates [23]. Brazilian strains have shown the existence of 15 ribotype patterns, 3 of which are associated most frequently with purpuric fever [24]. On amplification by long-PCR, the ribosomal operons of the non-typable strains of *H. influenzae* from Australia, have been found to be highly polymorphic for internal *HaeIII* sites [26]. In a recent study from Japan, long-PCR ribotyping of the strains did not allow any differentiation among the Hib isolates on digestion with *HaeIII*. On the other hand,

RAPD analysis of these strains showed two distinct patterns that were biotype-specific [27]. In the present study, the ribotypes obtained with *HaeIII* restriction endonuclease generated three banding profiles and were almost similar for the typable and non-typable isolates. Highly polymorphic bands were observed among the typable isolates with *EcoRI*, although non-typable isolates did not reveal much heterogeneity (Fig. 1, Table 1).

In the present study, the arbitrary primers AP1 and AP2 used for RAPD analysis are from the enterobacterial repetitive intergenic consensus sequences. These sequences are highly conserved and are able to show discrimination in many other organisms [33, 34]. In the present study, variations within the typable strains were observed by AP1 primer PCR fingerprinting. However, the non-typable *H. influenzae* demonstrated no differentiation with this primer. Primer AP2 was found to be more discriminatory and was able to show variations even within the serologically non-typable strains. Genotypic typing of the Indian *H. influenzae* isolates showed a high DI value by RAPD fingerprinting.

The data further reveal concordance of profiles with the isolates from blood, sputum and a few of the CSF samples from the respective patient groups by both ribotyping and RAPD-fingerprinting. This was in contrast to isolates from normal individuals, which presented random profiles.

In conclusion, the ability to identify strain variants appearing during persistent *H. influenzae* infections indicates that RAPD-fingerprinting is suitable for revealing genotypic diversity within serotypes. This typing method may be useful in the investigation of outbreaks of infection as it is quick to perform, requires modest effort and no previous genetic knowledge of the target organism is needed.

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