Outer-membrane-protein subtypes of *Haemophilus influenzae* isolates from North India

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Haemophilus influenzae serotype b and non-typable isolates from blood, cerebrospinal fluid, sputum and throat swabs of patients and carriers in North India were analysed by outer-membrane protein (OMP) profiling. OMP analysis could differentiate the samples into 18 different subtypes. The non-typable isolates were more variable than the serotype b samples. OMP subtypes 1–6 were found only among the serotype b isolates and subtypes 7–18 among the non-typable isolates, while subtypes 2 and 8 were exhibited by both. The OMP profiles of isolates from blood, cerebrospinal fluid and sputum are in complete agreement with their ribotypes and RAPD fingerprints. The present study demonstrates for the first time the subtyping of Indian *H. influenzae* isolates by an easy and less-expensive method that is applicable to developing countries like India.

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INTRODUCTION

Haemophilus influenzae is the causative agent of a variety of local and invasive infections in children and adults (Turk, 1984). Isolates of *H. influenzae* are divided into encapsulated strains, serotypes a–f, based on capsular polysaccharide antigenicity, and non-encapsulated strains, designated non-typable. *H. influenzae* type b (Hib) is responsible for invasive infections such as meningitis, epiglottitis and septicaemia. Non-typable *H. influenzae* (NTHi) strains cause otitis media, chronic bronchitis and pneumonia. NTHi can on occasion breach the epithelial barrier and cause septicaemia, endocarditis or pyogenic arthritis (St Geme, 1996).

Information regarding the incidence of diseases caused by *H. influenzae* and their severity, sequelae and complication rates is limited in India. Hospital-based data on *H. influenzae* infections are also rare. John *et al.* (1998) have reviewed and summarized information on diseases caused by *H. influenzae* in India covering more than three decades. They estimated that as many as 75–100 cases of pyogenic meningitis are caused by this organism per year per 100 000 children under 5 years of age. A study conducted by the Invasive Bacterial Infections Surveillance group also suggested that the burden of severe *H. influenzae* infection is substantial in India, accounting for about 30 % of the total bacterial meningitis cases in children. Therefore, for improved prevention and control of disease, it is necessary to know the types of strain circulating in the community.

Current vaccines against *H. influenzae* are effective only against strains possessing the type b polysaccharide (Hib).

Abbreviations: CSF, cerebrospinal fluid; Hib, *H. influenzae* serotype b; NTHi, non-typable *H. influenzae*; OMP, outer-membrane protein.

Although their widespread use in affluent countries has resulted in a decline in the incidence rate of Hib infections, there is a growing incidence of infections caused by non-type-b and non-typable strains of *H. influenzae*. Outer-membrane proteins (OMP) have been a major focus of research aimed at identifying conserved and antigenic surface components for inclusion in vaccines and for their ability to separate non-typable strains into subtypes corresponding to a particular epidemiological area.

The outer membrane of *H. influenzae* contains about 36 proteins, of which between six and eight make up the major protein content, with the remainder existing as relatively minor proteins (Loeb & Smith, 1980). The major OMPs include heat-modifiable 47 kDa P1 (Loeb, 1987), 39 kDa P2 porin (Munson *et al.*, 1983), 28 kDa P4 lipoprotein (Green *et al.*, 1991), 37 kDa P5 (Loeb & Smith, 1980), 16 kDa P6 or peptidoglycan-associated lipoprotein (Munson & Granoff, 1985) and the high-molecular-mass proteins HMW1 and HMW2 (Noel *et al.*, 1994). The minor OMPs include P6 cross-reacting protein (PCP, 15 kDa) (Deich *et al.*, 1990), OMP26 (26 kDa) (Kyd & Cripps, 1998), 80 kDa D15 (Loosmore *et al.*, 1997), 42 kDa protein D (Akkoyunlu *et al.*, 1991), transferrin-binding proteins of 85 and 100 kDa (Schryvers, 1989; Morton & Williams, 1990), haem:haemopexin-binding protein (Cope *et al.*, 1998), haemoglobin-binding proteins (Ren *et al.*, 1998) and several high-molecular-mass proteins ranging from 120 to 125 kDa. Some OMPs are consistently present in all strains of *H. influenzae*, while others vary among the typable and non-typable strains.

The preparation of sarkosyl-insoluble membrane proteins provides purified OMPs (Filip *et al.*, 1973) that form the basis of subtyping schemes defined for *H. influenzae* strains

(Barenkamp *et al.*, 1981; Granoff *et al.*, 1982; van Alphen *et al.*, 1983). This technique has been shown to be highly discriminatory and has been used with success to investigate putative outbreaks of infection with *H. influenzae*.

Subtyping of the present isolates based on molecular techniques has been reported earlier from our laboratory (Sharma *et al.*, 2002). An alternative to this was sought, since the methods were expensive and their application in a country like ours was not satisfactory. Thus, the present study was intended to subtype *H. influenzae* isolates from India by a less-expensive method based on differences in their OMP patterns.

METHODS

Study population. A total of 120 *H. influenzae* isolates was obtained from cerebrospinal fluid (CSF), blood, sputum samples and throat swabs of patients and healthy carrier population, of which 77 were Hib and 43 NTHi. Details of patients and numbers of samples screened have been given previously (Sharma *et al.*, 2002). The same samples were used for analysis in the present study.

Bacterial isolates. *H. influenzae* strains were grown on chocolate agar supplemented with haemin and NAD. The presence or absence of capsular polysaccharide was detected by slide agglutination using antisera to the capsular antigens a–f (Difco). Isolates were stored at –70 °C in brain heart infusion broth containing 5% glycerol.

Preparation of OMPs. Outer membranes were prepared by the method of Carlone *et al.* (1986) with a few modifications. This method involves lysis of the cells by ultrasonication. Chocolate-agar plates were inoculated with strains of *H. influenzae* and incubated so as to obtain 18 h growth. The growth was harvested with 2 ml 10 mM HEPES buffer per plate and transferred to centrifuge tubes that were centrifuged at 40 000 g for 30 min. The pellet was resuspended in 10 ml 10 mM HEPES buffer containing 5 mM PMSF. Sonication was done four or five times in a sonicator for 30 s with 30 s breaks in between (in an ice-bath). Intact cells and large debris were removed by centrifugation at 200 g for 30 min at 4 °C. The total membrane preparation was harvested from the supernatant by ultracentrifugation at 100 000 g for 1 h at 4 °C. The clear, gel-like pellet was resuspended in 1% sodium lauryl sarcosinate in 10 mM HEPES buffer for 30 min at room temperature and the detergent-insoluble fraction was harvested by centrifugation at 100 000 g at 4 °C for 1 h. The pellet was washed twice at 100 000 g at 4 °C for 1 h in HEPES containing PMSF. After washing, the pellet was suspended in 500 µl HEPES with PMSF and 50 µl aliquots were stored at –20 °C.

Separation of proteins by SDS-PAGE. Protein concentrations were estimated by the modified method of Lowry *et al.* (1951). SDS-PAGE was done by the method of Laemmli (1970) using a discontinuous buffer system with 15% (w/v) polyacrylamide as the resolving gel. Samples were prepared by adding 5× sample buffer to 1× final concentration and heating in a boiling water bath for 5 min. Low-molecular-mass standards (Roche) were included with each run. The gel was run at a constant current of 40 mA until the dye front just reached the bottom. The gel was then fixed in 12% trichloroacetic acid for 30 min, stained overnight in Coomassie brilliant blue, destained and finally photographed.

RESULTS

In the present study, the heterogeneity among *H. influenzae*

strains prevalent in North India was studied by OMP analysis. A total of 305 CSF samples and 41 blood samples from infants, throat swabs of 63 healthy children and adults and sputum samples from 25 adult patients were screened. Details of the samples and strains obtained were provided previously (Sharma *et al.*, 2002).

The OMP profile for each subtype when examined by SDS-PAGE showed 10–22 OMPs with molecular masses of approximately 15–100 kDa (Fig. 1). The 28 kDa P4 protein was conserved among both the typable and non-typable isolates, while the 16 kDa P6 protein, which elicits protective antibody against NTHi and has been reported in the literature to be conserved, was variable. It appears that P6 is present in some strains and absent in few. Several minor OMPs were also present in each subtype. OMP P2 was the most abundant, with a molecular mass of 36–42 kDa.

OMP analysis differentiated our set of strains into 18 different subtypes on the basis of variation in the major OMPs, with molecular masses ranging from 25 to 50 kDa and also from 14 to 20 kDa (Fig. 1), based on reproducible and clearly resolvable patterns. Variation in the OMP patterns was observed within and between isolates. Over 50% of Hib strains belonged to three predominant subtypes (subtypes 1–3; Table 1). Subtypes 7–18 were observed among the NTHi strains and subtypes 1–6 were present in Hib strains in our North Indian community. Subtype 8 (Fig. 1, lane 8), with two distinct major OMPs, of 36 and 34 kDa, was exhibited by one strain isolated from blood (Hib) and one strain isolated from the throat swab of an adult (NTHi) (Table 1). Similarly, subtype 2 was common to both Hib strains isolated from CSF and NTHi strains isolated from throat swabs of children (Table 1).

In the present study, three Hib strains from blood showed the same subtype (subtype 6); a single Hib isolate from blood showed subtype 8 in OMP profile analysis (Table 1). Though

Fig. 1. OMP subtypes of Hib and NTHi isolates. Lane numbers (1–18) indicate representative OMP subtypes. The subtypes were categorized on the basis of variations in the major OMPs with molecular masses ranging from 25 to 50 kDa and also from 14 to 20 kDa. Lane M, molecular mass marker.

Table 1. Subtype distribution of Indian *H. influenzae* isolates by OMP analysis

All Hib strains were obtained from infants. TS, Throat swab. OMP subtypes correspond to the lanes in Fig. 1.

OMP subtype	Hib		NTHi	
	Source	No. of isolates	Source	No. of isolates
1	CSF	18	—	0
2	CSF	17	TS child	2
3	CSF	25	—	0
4	CSF	5	—	0
5	CSF	8	—	0
6	Blood	3	—	0
7	—	0	TS child	3
8	Blood	1	TS adult	1
9	—	0	TS child	2
10	—	0	TS adult	1
11	—	0	Sputum	8
12	—	0	TS adult	2
13	—	0	TS adult	1
14	—	0	TS child	6
15	—	0	TS adult	3
16	—	0	TS child	9
17	—	0	TS adult	2
18	—	0	TS child	3

they were from serotype b, the CSF isolates also showed polymorphism. Among the 18 CSF samples of OMP subtype 1, only six isolates previously showed similar ribotypes and RAPD profiles. Of the NTHi isolates, eight strains isolated from sputum samples of patients with chronic bronchitis (Table 1) exhibited similar profiles (subtype 11) in OMP analysis when examined in SDS-PAGE. However, NTHi isolates from throat swabs of the healthy carrier population were distributed randomly over the various subtypes, as in our previous study.

DISCUSSION

Our previous study on the epidemiology of *H. influenzae* using molecular approaches showed a high prevalence of infections associated with *H. influenzae* in our community. In the present investigation, we have studied the epidemiology of *H. influenzae* strains by protein-based methods. This is the first report from India regarding *H. influenzae* typing by OMP analysis. Subtyping of *H. influenzae* by protein analysis has been done for strains from North America, Europe and the UK (Pennington & Freebairn, 1989; Jordens *et al.*, 1993).

OMP analysis differentiated our set of strains into 18 subtypes, suggesting diversity among *H. influenzae* strains in our community. OMP analysis of *H. influenzae* isolates from North America revealed 21 distinct OMP types, with 89 % of strains belonging to only six OMP types, but there

was a lack of correlation between OMP profiling and biotyping (Barenkamp *et al.*, 1981). In Western Europe, OMP type 1 is reported to be significantly more common, though OMP 11 was more prevalent in Iceland (van Alphen *et al.*, 1987). Similarly, we also observed some predominant subtypes (1–3) and some subtypes common to Hib and NTHi isolates (2 and 8) (Table 1).

In a study from The Netherlands of 80 *H. influenzae* strains isolated from CSF of patients with meningitis, OMP analysis revealed that 84 % of the isolates had identical major OMP patterns (subtype 1). Of the remaining isolates, four different PAGE patterns were observed, two of which closely resembled subtype 1 (van Alphen *et al.*, 1983). In this study, the OMP patterns observed were based on differences in the position and electrophoretic mobility of the major OMPs a (47 kDa), c (40 kDa), d (37 kDa) and e (30 kDa). The Dutch strains therefore did not show much variability in their OMP patterns. In comparison, our Hib strains from CSF were found to be more heterogeneous and gave five different OMP patterns (Table 1).

A study from Pakistan analysed clonal diversity of 95 *Haemophilus* isolates from blood of children with lower respiratory tract infection by OMP analysis. Of the *H. influenzae* isolates, 61 (64 %) were Hib and 34 (36 %) were NTHi; 95 % of the Hib isolates were members of a single clonal group, as defined by SDS-PAGE OMP profile analysis, multilocus enzyme electrophoresis and biotype analysis. This clone is rarely observed among Hib strains recovered from patients with invasive type-b disease in the USA or Europe. The non-typable isolates from Islamabad were also clonally restricted; nine clonal groups were found among 34 isolates, with just five clonal groups accounting for most (82 %) of the strains (Weinberg *et al.*, 1989). In contrast, our strains did not reveal any clonal subtype by OMP analysis.

Our study has also shown that the NTHi strains prevalent in our community have diverse OMP profiles, which suggests that NTHi strains are more heterogeneous in nature compared with Hib strains. A study by Aparicio *et al.* (1996) also showed that Hib strains had a strong clonal structure, in contrast to NTHi strains. Hib strains showed one pattern with isoenzymes, OMP and ribotyping techniques. Only PFGE allowed differentiation between several Hib patterns, although all of them were closely related. All methods showed a great variety of patterns with NTHi strains.

An important finding of our study is that isolates from blood, CSF and sputum samples of patients showed complete agreement in their profiles produced by molecular approaches (Sharma *et al.*, 2002) and OMP analysis. In the OMP analysis, the same three isolates from blood that presented ribotype A with *EcoRI* and RAPD type a with primers AP1 and AP2 in our previous study showed OMP subtype 6, while the strain of ribotype D and RAPD type d showed OMP subtype 8. Six of the Hib isolates from CSF samples that gave ribotype A with *EcoRI* and *HaeIII* and RAPD type a with both primers clustered into OMP subtype 1. Among the NTHi isolates, OMP subtype 11 was generated

by all eight isolates from sputum samples of patients with chronic bronchitis that were of ribotype A with *Hae*III and RAPD types g and k with primers AP1 and AP2, respectively. Therefore, OMP subtyping, which is cheaper, reproducible and more sensitive to strain variations, provides an important tool for epidemiological studies in developing countries such as India.

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