Role of biofilm formation in the persistent colonization of *Haemophilus influenzae* in children from northern India

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The human nasopharynx is a major ecological niche for *Haemophilus influenzae* colonization. Establishment of infection is critically dependent on the persistence of bacteria in the nasopharynx. Various factors are presumed to mediate this persistence and the influence of biofilm formation has been under scrutiny for a long time. In a prospective population-based study, the nasopharyngeal colonization pattern of 250 children <2 years old was traced to gain further insights into the phenomenon. The association between biofilm formation and persistence was delineated by quantitative biofilm assay, while the true nature of biofilm formers was further evaluated by electron microscopy studies. *H. influenzae* isolates obtained in this study, when analysed by phenotypic and genotypic means, revealed a clonal distribution of strains within the population. The biofilm formation ability of the isolates was found to be significantly associated with bacterial persistence ($P<0.001$). The isolates having biofilm formation ability were found to be 7.1 times more likely to persist in the nasopharynx than non-biofilm formers. This study provides direct evidence indicating the intricate relationship between biofilm formation and the persistence of bacteria. Our results emphasize the need to evaluate the potential for biofilm formation before designing preventive and therapeutic strategies.

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INTRODUCTION

The human nasopharynx is densely colonized by a broad variety of microbes including commensal bacterial flora, as well as pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella* (Todd, 1984; Faden *et al.*, 1997). Nasopharyngeal colonization precedes invasion, but disease occurs only in a small percentage of persons who are colonized. The pattern of nasopharyngeal colonization is a cryptic event in pathogenesis, and understanding the mechanisms of colonization of the nasopharyngeal mucosa has become a core issue in the study of respiratory infections (Raymond *et al.*, 2001).

Persistence in the host environment is a ploy used by the pathogen to ward off the attacking forces from the host. This act of persistence is brought about by an adaptive event termed as biofilm formation, a defence strategy employed by the micro-organism to evade the hostile environment within the host (Stanley & Lazazzera, 2005). A biofilm can be defined as a group of bacteria growing as a community and encased in a self-produced polymeric matrix. By adopting

this mode of living the micro-organism succeeds in evading recognition by the host, as well as becoming impermeable to various chemical defences (Yang *et al.*, 2008).

Various pathogens inhabiting the respiratory tract, such as *Pseudomonas* in cystic fibrosis patients (Landry *et al.*, 2006) and *Bordetella pertussis* (Sloan *et al.*, 2007), are shown to be successful in adopting the biofilm formation as their lifestyle. Numerous studies have investigated *H. influenzae* colonization among healthy adults, cystic fibrosis and chronic obstructive pulmonary disease patients (Stanley & Lazazzera, 2005; Starner *et al.*, 2006; Murphy *et al.*, 2004). But since nasopharyngeal colonization starts in the first year of life (Vives *et al.*, 1997), concrete efforts are needed to understand the influence of various factors. In this context, a prospective study of children under 2 years old was carried out to elucidate the dynamics of persistent bacterial colonization in the nasopharynx, and to determine the index of association between bacterial persistence and biofilm formation.

METHODS

Child enrolment. The study comprised 250 children in the 2–24 months age group who were enrolled from rural areas, urban slums

Abbreviations: RAPD, random amplified polymorphic DNA; SEM, scanning electron microscopy.

and city areas of northern India during the period January 2006 to July 2006, after obtaining informed consent from the parents. Children who had been administered any antibiotic were excluded from the study. The complete sociodemographic structure of this study population has been described previously (Sekhar *et al.*, 2009). Nasopharyngeal sampling was repeated every 4 weeks within the same population who had carriage of *H. influenzae* in the previous sampling.

Microbiological analysis. Nasopharyngeal specimens were obtained with a calcium alginate swab (Calgiswab, Pur-Wraps) and were transported in skimmed milk, tryptone, glucose, glycerol (STGG) medium. These swabs were later streaked onto chocolate agar plates with 300 µg bacitracin ml⁻¹ within 24 h of swab collection. The colonies with morphology typical of *H. influenzae* were identified based on X, V factor requirements. The confirmed species of *H. influenzae* were frozen at -70 °C in trypticase soy broth with 20 % glycerol.

Serotyping and biotyping. Serotyping was carried out on each positive culture using polyvalent antisera against *H. influenzae* (Difco Laboratories). Capsulate *H. influenzae* were further confirmed by *bexA* gene amplification (Van Ketel *et al.*, 1990). Biotyping of all the *H. influenzae* isolates was performed by assessing their ability to produce urease, indole and ornithine decarboxylase (Kilian, 2005; Gratten, 1983; Sottnek & Albritton, 1984).

DNA isolation. *H. influenzae* isolates were subcultured onto chocolate agar plates. DNA was isolated following the protocol of Loos *et al.* (1989), with slight modifications.

Random amplified polymorphic DNA (RAPD) analysis. RAPD analysis was performed with the DNA of *H. influenzae* using the following primers (Akopyanz *et al.*, 1992): P1, 5'-GGTTGG-GGAGAATTGCACG-3'; P2, 5'-AAGTAAGTGACTGGGGTGAGCG-3'; P3, 5'-GTAGACCCGT-3'. The PCR program consisted of a denaturation phase of 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 32 °C and 2 min at 72 °C. The discriminatory ability of RAPD typing was determined by calculating the numerical discrimination index using the method of Simpson (1949).

Quantification of biofilm formation. Quantification of biofilm formation was carried out by microtitre plate assay as described by O'Toole & Kolter (1998) with slight modifications.

Visualization of biofilms by scanning electron microscopy (SEM) and transmission electron microscopy. Preparation of biofilms on solid substrates was carried out by methods described by Thurnheer *et al.* (2003). Sterile Millipore filters (GSW 2500; Millipore) were placed on the surface of chocolate agar plates and incubated with a known number (1×10^7) of *H. influenzae* in brain heart infusion broth. The filters with bacteria were incubated overnight at 37 °C in 5 % CO₂ and 95 % relative humidity. Biofilms formed on the filter substrates after 24 h of incubation were prepared for further analysis by a standard chemical fixation protocol.

Statistical analysis. Quantitative biofilm assay data were compared using a non-parametric Mann-Whitney test. Logistic regression analysis was performed to assess the influence of epidemiological factors and biofilm formation on the persistence of *H. influenzae* in nasopharynx.

RESULTS AND DISCUSSION

H. influenzae colonization in <2-year-old children

This study involved 120 children from rural areas, 65 from urban slums and 65 from city areas. The mean age of

children was 12.8 months. Contact of children with one another occurred in just a few cases (1.2 %) and attendance of children in day care centres was also very low (1 %). None of the children amongst our study population had received type b *H. influenzae* vaccine or the pneumococcal conjugate vaccines. The bacterial colonization in these children was 19.2 % (48/250) in the first sampling, followed by a gradual decrease in colonization in the subsequent samplings revealing the transient nature of colonization in the nasopharynx (Fig. 1). In the current study it was observed that only 30 % (14 of 46) of type b isolates were detected on two or more occasions in the nasopharynx. A total of 89 % (41 of 46) of type b isolates colonized for 2 months or less, while 47 % (25 of 53) of non-typable *H. influenzae* persisted in the nasopharynx for more than 2 months. These mean carriage durations were similar to the observations reported elsewhere (Raymond *et al.*, 2001; Spinola *et al.*, 1986). The difference in carriage duration between type b *H. influenzae* and non-typable *H. influenzae* may be associated with factors such as adherence, biofilm formation ability and other host factors that are not yet well characterized.

Serotyping and biotyping of *H. influenzae* isolates

Serotyping of the study isolates in the first sampling showed a majority of type b (66.6 %). From the second sampling onwards the trend was reversed with the majority of the isolates being non-typable (57 %). In the third sampling 66.6 % of these isolates were non-typable. The last samplings yielded 100 % non-typable *H. influenzae* (Fig. 1). Biotype 1 was predominant at 41 %, followed by biotypes 3, 2 and 4 at 21, 19 and 11 %, respectively.

Diversity of non-typable *H. influenzae* in comparison to type b isolates

With the aim of defining the genetic relationship of the study isolates, a RAPD analysis using three primers was

Fig. 1. Distribution pattern of *H. influenzae* isolates during repeated samplings and their cluster patterns. White bars, Type b; grey bars, non-typable *H. influenzae*; ●, cluster.

Fig. 2. (a) Strains depicting the variable RAPD patterns. Lanes 1–10, RAPD patterns of different strains; NC, negative control; M, molecular mass marker. (b) RAPD profiles of the strains isolated from repeated samplings. Lanes 1–6, RAPD patterns in repeated samplings of the same subject; M, molecular mass marker.

performed. The samples, when analysed on a 1.5 % agarose gel, showed 3–6 bands with a fragment length distribution in the range of 0.1–1.5 kb (Fig. 2a). Persistent strains were found to have a similar RAPD pattern on repeated samplings. Furthermore, these RAPD profiles were highly reproducible (Fig. 2b) and had a high discrimination index of 0.94. More strains with similar genotypes were defined

Table 1. Subtype distribution of isolates used in the quantitative biofilm assay

	Biotype				RAPD										
	1	2	3	4	A	B	C	D	E	G	H	I	J	K	
Persister															
Hib isolates	5	2			1	1	2	2	1						
NTHi isolates	3	4	1			1	1	1		1	1	1	1	1	
Non-persister															
Hib isolates	4	1	1	1	1	1	1	3	1						
NTHi isolates	2	5	1		1	1	1	1		2	1			1	

Hib, Type b *H. influenzae*; NTHi, non-typable *H. influenzae*.

as a cluster. In the first sampling type b isolates based on their DNA fragment patterns were grouped into 5 clusters, while non-typable isolates were grouped into 12 clusters. In the second sampling among type b strains there were two clusters, while the non-typable isolates were grouped as seven clusters. In the third sampling a single cluster was depicted among type b isolates while non-typable isolates had six distinct patterns. The fourth sampling had a single cluster of type b and five clusters in the non-typable group. In the fifth and sixth samplings there were four clusters among the non-typable strains. Thus, a high genetic heterogeneity was demonstrated among non-typable *H. influenzae* in comparison to type b isolates in the studied population. This is in accordance with observations made in other studies (Gomez-De-Leon *et al.*, 2000; Smith-Vaughan *et al.*, 1996).

Fig. 3. (a) Quantitative ability of biofilm formation among *H. influenzae* isolates. The bars represent means of three experiments performed in quadruplicate. Error bars represent SD. (b) SEM of a strain showing biofilm formation (the inset shows the characteristic organized microcolony formation of persistent bacteria). (c) SEM of a strain showing no biofilm formation.

Biofilm formation ability in *H. influenzae* clinical isolates

A quantitative biofilm assay was performed with 15 strains (7 type b and 8 non-typable) chosen from the group showing a longer duration of colonization (≥ 8 weeks) and 15 other strains (7 type b and 8 non-typable) from the group with a shorter duration of colonization (< 8 weeks period). Four children taking part in the study developed otitis media and the isolates obtained from them were non-typable. When the serotype, biotype and RAPD patterns of study isolates were compared in order to differentiate the biofilm-forming capacity of these clusters, it was observed that two clusters (I and J) were exclusively present among the non-typable isolates in the persister group (Table 1). The biofilm formers had a mean $\pm$ SD 1.6 ± 0.2 , while among the non-biofilm formers the value was 0.7 ± 0.4 . The difference in mean values between the groups was highly significant ($P < 0.05$) (Fig. 3a). The strains showing higher median optical density values in the quantitative biofilm assay when analysed by SEM, showed a characteristic microcolony formation (Fig. 3b) and extruded exopolysaccharide, which is a hallmark event in biofilm formation (Webster *et al.*, 2004). While in comparison the non-biofilm-forming isolates showed individual cells upon

the SEM (Fig. 3c). The viability of isolates was found to be intact for all the strains obtained from plates when assessed after the quantitative assay. This ruled out the possibility of any loss of viability during the post-exponential phase of growth of the bacteria. The presence of different (2–5 %) concentrations of glucose, sucrose and xylose was found to have no significance with respect to biofilm formation. Furthermore, on logistic regression analysis of the factors contributing towards the persistence of *H. influenzae* in the nasopharynx, biofilm formation was found to be a significant factor contributing towards persistence (Table 2). The use of uniform inoculum in the quantitative biofilm assay nullified the role of bacterial concentration in influencing the outcome of the quantitative biofilm assay. It corroborates the evidence that biofilm formation is an intricate phenomenon with its focus featured on events other than the mere growth of the bacteria. This brings about the hypothesis of the existence of factors that produce a tangible change in the mode of living of the pathogen (Hong *et al.*, 2007), and the role of various surface moieties such as phosphocholine and sialic acid, and confer an adaptive advantage during biofilm formation (West-Barnette *et al.*, 2006). However, other factors, such as the local immune response, may also play a role in persistence and have not been addressed in our study.

Table 2. Factors analysed for the persistence of *H. influenzae* in the nasopharynx of children from northern India

Risk factor	Odds ratio*	95 % CI (lower)	95 % CI (upper)	P value
Age				
≥ 12	0.5	0.1	1.6	0.2
< 12	1	Reference	Reference	
Gender				
Male	0.5	0.2	1.4	0.2
Female	1	Reference	Reference	
Overcrowding (persons/room)				
> 2	0.4	0.1	1.8	0.2
≤ 2	1	Reference	Reference	
Housing type				
'Katcha' (temporary)	0.5	0.1	1.4	0.2
'Pucca' (permanent)	1	Reference	Reference	
Family member				
> 8	0.5	0.2	1.6	0.2
≤ 8	1	Reference	Reference	
Season				
Winter	0.6	0.2	1.8	0.3
Summer	1	Reference	Reference	
Biofilm formation				
Biofilm former	7.1	2.7	18.7	< 0.0
Non-biofilm former	1	Reference	Reference	
<i>H. influenzae</i> type				
NTHi	1.2	0.1	13.1	0.8
Hib	1	Reference	Reference	

CI, Confidence interval; Hib, type b *H. influenzae*; NTHi, non-typable *H. influenzae*.

*Based on logistic regression model that included all variables.

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