Genetic Diversity of Invasive Strains of Haemophilus influenzae Type b before and after Introduction of the Conjugate Vaccine in Italy

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We determined the genotypes of 95 invasive Haemophilus influenzae type b (Hib) strains collected before and after introduction of widespread Hib vaccination in Italy. No substantial change in genetic diversity was highlighted by pulsed-field gel electrophoresis. However, an upward temporal trend in proportion of strains possessing multiple copies of the capsulation b locus was detected (P = .03).

Routine use of Haemophilus influenzae type b (Hib) conjugate vaccines has dramatically decreased the incidence of invasive Hib disease in developed countries. Despite the effectiveness of the vaccine, an increase in the incidence of Hib disease has recently been observed in the United Kingdom and in The Netherlands [1, 2]. Predisposing host risk factors or the use of less immunogenically combined vaccines containing the acellular pertussis component have been associated with cases of Hib conjugate vaccine failure [3, 4]. Recently, the focus of investigations on Hib has moved from host to bacterial properties. Several reports have emphasized the need to monitor the impact of vaccines on the circulating Hib population in which particular clones might successfully evade host immune response [5, 6]. Moreover, it has been suggested that changes of capsule expression as result of amplification of capsulation response [5, 6].

In Italy, Hib vaccination (consisting of 3 doses at 3, 5, and 11 months of age) has been included in the national vaccination program since 1999. Hib vaccine coverage by 24 months of age was estimated to be 20% in 1998, 55% in 2000, 84% in 2002, 87% in 2003, and 94% in 2004 (Italian Ministry of Health http://www.ministerosalute.it/linksanita/malinf.htm). To evaluate the burden of H. influenzae invasive disease and monitor the impact of the vaccination program, a laboratory-based surveillance study has been conducted in a sample of Italian regions since 1997 [8]. During 1997–2003, a total of 225 confirmed cases of invasive Hib disease were reported. One hundred fifty-seven of these cases (69.8%) were culture confirmed, whereas 68 (30.2%) were positive for Hib antigen detection in CSF specimens. The impact of vaccination on the incidence of invasive Hib disease in Italy was comparable with that of other industrialized countries, leading to a decrease in the annual incidence from 0.27 cases/100,000 persons to 0.02/100,000 persons in the total population and from 4.78/100,000 persons to 0.44/100,000 persons among children aged <5 years (P< .0001).

In the present study, the genetic structures of invasive Hib strains collected before and after Hib vaccination had been included in the national program (1997–2003) were investigated. A total of 95 consecutive Hib strains were analyzed by assessing their genetic relationships using PFGE and by determining the number of copies of the cap b locus.

Methods. Ninety-five Hib strains isolated during the period of June 1997 through December 2003 from patients with invasive disease detected through active surveillance were sent to the National Reference Laboratory at Istituto Superiore di Sanità (ISS), Rome, Italy, and were included in this study. All isolates were identified as serotype b by PCR capsular genotyping [9]. The 95 isolates were examined using PFGE (in accordance with procedures described previously [8]) after digestion of the genomic DNA with the Smal restriction enzyme. DNA fragments were analyzed using the unweighted pair group mean association clustering method and Dice’s coefficient with the Diversity Database Fingerprinting Software, version 2 (BioRad). One Hib isolate (strain 40F) belonging to the clone endemic in Italy [10] was included in PFGE analysis. For each isolate, the copy number of the cap b locus was determined by Southern blot analysis on the basis of the size of the restriction fragments after digestion of the chromosome with KpnI and Smal restriction enzymes, in accordance with procedures described elsewhere[7]. In Hib strain 237, the serum concentration of IgG antibodies against Hib capsular polysaccharide polyribosyl ribitol phosphate (PRP) was determined using the BINDAZYME Human Anti Haemophilus Influenzae Enzyme...
Table 1. Amplification status of the \textit{Capsulation} b locus in 95 invasive \textit{Haemophilus influenzae} type b (Hib) strains isolated before (1997–1998) and after (1999–2003), when Hib vaccination was included in the National Program.

<table>
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<tbody>
<tr>
<td>2</td>
<td>40 (66.7)</td>
<td>11 (50)</td>
<td>4 (44.4)</td>
<td>1 (25)</td>
<td>16</td>
</tr>
<tr>
<td>3–5</td>
<td>20 (33.3)</td>
<td>11 (50)</td>
<td>5 (55.6)</td>
<td>3 (75)</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>22</td>
<td>9</td>
<td>4</td>
<td>35</td>
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\textbf{NOTE.} Temporal variation in proportion of multiple-copy strains was statistically significant by the Cochran-Armitage exact test for linear trend, \(P = .03\).

Immunoblotting Kit (The Binding Site, Birmingham, UK), according to the manufacturer’s instruction. Proportions were compared using the \(\chi^2\) test or Fisher’s exact test. The Cochran-Armitage exact test for linear trend was used to compare the proportion of strains with multiple copies over time (1997–1998, 1999–2000, 2001–2002, and 2003).

\textbf{Results.} Of the 95 Hib strains analyzed, 60 were obtained during the years 1997–1998, before Hib vaccination was included in the national program, and 35 strains were isolated during the years from 1999–2003, after Hib immunization had become routine (11 strains in 1999, 11 in 2000, 5 in 2001, 4 in 2002, and 4 in 2003). Strains had been isolated from various clinical specimens, as follows: CSF, 61 isolates; blood, 33 isolates; and synovial fluid, 1 isolate. The age of patients was available for all but 5 patients. Seventy-eight strains were recovered from children aged <5 years (median age, 11.0 months; range, 2.0–54.0 months), and 12 were recovered from patients aged ≥5 years (median age, 51.5 years; range, 6.0–89.0 years). Of the 78 strains obtained from children, 2 were collected from those who had previously received 1 dose of conjugate Hib vaccine, and 2 were collected from patients who had experienced true vaccine failure, as previously defined [3].

By PFGE, the 95 Hib strains yielded 28 distinct restriction patterns. Five patterns (17.9%) included multiple isolates, whereas the remaining 23 (82.1%) consisted of 1 or 2 isolates. One pattern was shared by 44 strains (46.3%) and was indistinguishable from the profile of the strain 40F belonging to the major invasive Hib clone that has been endemic in Italy since 1994 [10]. Clustal analysis of the PFGE patterns showed that most isolates (86 [90.5%] of 95), including isolates from infants who had previously received conjugate Hib vaccine, displayed high genetic homology (coefficient of similarity, \(\geq 0.80\)) (data not shown). The 12 strains recovered from patients aged ≥5 years were distributed into 8 patterns, of which 6 gathered in the major clonal group. No association was found between specific pattern and site of isolation (CSF or blood).

When the copy number of the \(\text{cap} b\) locus was determined, 56 strains (58.9%) exhibited hybridization signals at the expected position for the 2-copy arrangement of the locus, and 39 strains (41.1%) showed hybridization bands at the expected position for 3-copy (20 strains), 4-copy (9 strains), or 5-copy (10 strains) arrangements. The proportion of multiple-copy isolates, harboring \(\geq 3\) repeats, steadily increased during the study period, ranging from 33.3% in 1997–1998 to 75% in 2003 (table 1). Despite the small number of isolates collected during 2001–2003, the observed temporal variation in proportion of multiple-copy strains was significant (\(P = .03\)). Moreover, grouping isolates into 2 periods—before (1997–1998) and after (1999–2003) Hib vaccination was included in the national program—the proportion of multiple-copy isolates was significantly higher in the years 1999–2003 (19 [54.3%] of 35) than in the years 1997–1998 (20 [33.3%] of 60; \(P = .046\)). Multiple-copy strains were found more frequently among isolates from blood (16 [48.5%] of 33) than among those from CSF (22 [36.0%] of 61), although this finding was not statistically significant. This result was in agreement with previous observations showing that presence of multiple-copy strains was associated with disease other than meningitis [7].

Interestingly, 3 of the 4 Hib strains isolated from vaccinated

Table 2. Clinical data of vaccinated children with \textit{Haemophilus influenzae} type b (Hib) invasive disease and characterization of isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time of isolation</th>
<th>Age of patient, months</th>
<th>Clinical presentation of patient(^a)</th>
<th>Site of isolation</th>
<th>No. of vaccine doses</th>
<th>Risk factor</th>
<th>Anti-PRP g/mL</th>
<th>No. of copies of (\text{cap} b) locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi 193</td>
<td>July 2000</td>
<td>3</td>
<td>Meningitis</td>
<td>CSF</td>
<td>1</td>
<td>Not known</td>
<td>...</td>
<td>2</td>
</tr>
<tr>
<td>Hi 227</td>
<td>September 2002</td>
<td>5</td>
<td>Cellulitis</td>
<td>Blood</td>
<td>1</td>
<td>Not known</td>
<td>...</td>
<td>3</td>
</tr>
<tr>
<td>Hi 229</td>
<td>March 2003</td>
<td>11</td>
<td>Meningitis</td>
<td>Blood</td>
<td>2(^b)</td>
<td>Premature (35 weeks)</td>
<td>...</td>
<td>3</td>
</tr>
<tr>
<td>Hi 237</td>
<td>September 2003</td>
<td>11</td>
<td>Cellulitis</td>
<td>Blood</td>
<td>2(^b)</td>
<td>None</td>
<td>0.55(^c); 0.31(^d)</td>
<td>5</td>
</tr>
</tbody>
</table>

\textbf{NOTE.} \(\text{cap}\), capsulation; PRP, polyribosyl ribitol phosphate.

\(^a\) All patients were discharged alive.

\(^b\) True vaccine failure.

\(^c\) Serum samples obtained at onset of Hib disease.

\(^d\) Convalescent-phase serum sample.
children with invasive disease harbored multiple copies of the \textit{cap} \textit{b} locus (table 2). In particular, 2 strains had 3 copies, and 1 strain contained as many as 5 copies. Considering the clinical data for the patients, the most common risk factor, prematurity, was present in only 1 child [3]. For the patient from whom the 5-copy strain had been isolated, both acute- and convalescent-phase serum samples had an anti-PRP antibody concentration $>0.15 \mu g/mL$ but $<1 \mu g/mL$ (the purported short-term and long-term protective levels), suggesting that both factors—suboptimal antibody response and amplification of the \textit{cap} \textit{b} locus—might have played a role in the failure.

**Discussion.** In the era of Hib conjugate vaccines, careful analysis of circulating Hib strains is essential for prompt detection of any change in the properties of bacteria, enabling particular clones to overcome the host's immune response. In this study, we characterized invasive Hib strains collected before (1997–1988) and after (1999–2003) Hib vaccination had been included in the national immunization program.

To investigate the genetic diversity of our isolates, we used PFGE, a powerful discriminatory tool for distinguishing between Hib strains. Clustal analysis results demonstrated that most isolates appeared to be strongly related genetically. Contrary to previous reports [6, 11], neither increased genetic diversity of Hib strains isolated from children nor the disappearance of individual clones was observed after the routine immunization of infants against Hib was established.

However, when we looked at changes in capsule genes, a statistically significant increase in the proportion of multiple-copy isolates was observed during the study period. Although the very low number of strains isolated in recent years can be considered a limit, we observed a steady trend that requires great attention. To date, only a few studies have been performed on the amplified state of the \textit{cap} \textit{b} locus in Hib isolates circulating during the postvaccination era [7, 12]. The results of this study suggest that vaccine pressure may be positively selecting for strains that harbor amplified \textit{cap} \textit{b} sequences. We speculated that serum rich in antibodies to capsule polysaccharide as a result of vaccination might play a role in such a selection. Considering the suggestion that the amplified state is a contributory factor in some cases of Hib conjugate vaccine failure in children [7], the finding that the majority of strains currently circulating in Italy, although few, possess multiple repeats of \textit{cap} \textit{b} locus, should be regarded with concern. An investigation of each future case of invasive Hib disease is necessary to determine whether this trend will continue.

**Acknowledgments**

We would like to acknowledge all members of the \textit{Haemophilus influenzae} study group: Annalisa Castella, Stefania Orecchia, Angela Ruggenini Moiraggi, and Carla Zotti (Regione Piemonte); Valter Carraro and Iole Caola (Provincia Autonoma di Trento); Antonio Ferro (Regione Veneto); Pietro Crovari, Marina Lemmi, and Giorgio Leprotto (Regione Liguria); Emanuela Balocchini, Paolo Bonanni, Patrizia Pecile, Licia Pecori, and Alessia Tomei (Regione Toscana); Vittorio Pagano and Renato Pizzuti (Regione Campania); Salvatore Barbuti and Maria Chiromma (Regione Puglia).

We are very grateful to Antonino Bella and Marco Massari for the help provided in performing statistical analysis.

**Financial support.** The Italian Ministry of Health, Target Project no. 4AIF.

**Potential conflicts of interest.** All authors: no conflicts.

**References**