An Epidemiological Study of *Haemophilus influenzae* at a Brazilian Day Care Center

Bonifácio da Silva M.E. and Marin J.M. *University of São Paulo, SP, Brazil*

Day care centers are a relatively new phenomenon in Brazil that bring together large numbers of young children susceptible to contagious diseases. *Haemophilus influenzae* (Hi) is an important infection in the age range of those attending day care centers. In the present study, the carriage rate of *Haemophilus influenzae* was identified in 38 day care attendees age 6 to 37 months, and 23 staff members, at a day care center in Ribeirão Preto-São Paulo, in 1997. To identify the carriers, two nasopharyngeal swabs were collected; one in July and one in December. The rate of *H. influenzae* carriers among the children was 77%. Only 2 of 23 staff members (9%) had Hi. Among the children, there were 58 isolates in the two sampling periods; 6 of the Hi were serotype b, 1 was serotype e, and 48 isolates were non-typeable. Two were identified as *H. parainfluenzae*. One adult had a non-typeable Hi and 1 had *H. paraphrohaemolyticus*. Three of the 6 children with type B had received a conjugate vaccine against *H. influenzae* type b, but they still carried this bacterium in the nasopharynx (50%). Forty ribotype patterns were found among the isolates, showing a high exchange rate of nontypeable *H. influenzae* carriers. The results indicate that, because of the high and changing biotype of *Hi* carriage, day care centers should be carefully monitored as potential point source of HI disease in the community.

Key Words: *Haemophilus influenzae*, day care, colonization.

Haemophilus influenzae (Hi) is a Gram negative coccobacillus which asymptomatically colonizes the nasopharynx of healthy individuals and, occasionally, causes systemic disease and mucous membrane infections [1]. Strains of *Hi* are classified according to their polysaccharide capsule. There are six structurally and antigenically distinct capsular serotypes designated from a to f, and nonencapsulated (nontypeable) strains [2].

More than 90% of systemic diseases are caused by *Hi* type b (*Hi*b). The availability of a vaccine based on the type b capsular antigen has dramatically reduced the incidence of such diseases in Europe [3], North America [4], and, more recently, in developing countries like Gambia [5]. The incidence of diseases caused by nonencapsulated *Hi* is still significant [6].

Nontypeable *Hi* (NT*Hi*) is a frequent cause of local infections such as otitis media, conjunctivitis, and sinusitis in children [7]. Some data suggest that NT*Hi* is a significant cause of invasive diseases, such as pneumonia, in children in developing countries [7] and pneumonia, septicemia, and meningitis in adults [8]. Although this pathogen is associated with a variety of infections, it is also frequently isolated from the upper respiratory tract of healthy children with reported carrier rates of up to 60% [9]. The significance of nasopharyngeal colonization by NT*Hi* is unclear, but colonization has been shown to be associated with an increased risk of developing acute otitis media and conjunctivitis in children attending day care centers [10].

Nasopharyngeal colonization with *Hi*, which precedes *Hi* infection and disease, is a dynamic process [11]. A possible explanation for prolonged *Hi* carriage

Received on 19 January 2001; revised 4 October 2001.
Address for correspondence: Dr. José Moacir Marin. Departamento de Morfologia, Estomatologia e Fisiologia, Universidade de São Paulo Campus Ribeirão Preto, Av do Café S/N° Campus USP, CEP 14040-904, Ribeirão Preto, São Paulo, Brazil.

The Brazilian Journal of Infectious Diseases 2001;5(5):260-268

1413-8670

includes the known ability of the bacteria to persist intracellularly [12], the induction of a weak inflamatory response by *Hi* carriage [13], and the presence of antigenic variation among colonizing *Hi* strains [14].

In Brazil, until recently (1999), *Hi*b vaccination was not part of the national immunization program, even though some cities widely use the *Hi*b vaccine [15].

The aims of the present six month day care population-based study were to examine the carriage rate of *Hi* in the nasopharynx of healthy children 6 to 37 months of age and their caretakers; to identify the biotypes and serotypes of the isolates; to assess possible relationships among isolates by ribotyping with the pKK3535 plasmid (rRNA operon); and to investigate the effect of vaccination against *Hib* on nasopharynx colonization by *Hib*.

Material and Methods

Sample collection

Samples were collected in July and December, 1997, at the Carochinha, a day care center on the São Paulo University Campus, Ribeirão Preto. Attendees were grouped by age into three modules: Pink - infants (aged 6 to 20 months), Green - toddlers (aged 19 to 37 months) and Blue - preschool group (aged 33 to 66 months). Due to the day care work schedule, each module was subdivided into four subgroups of 5 to 7 children and 1 or 2 staff members were responsible for alimentation, recreation, and hygiene in each subgroup. Only the Pink (subgroups A, B, C, D) and Green (subgroups E, F, G, H) modules were evaluated in this study. *Hi* colonization was investigated in 38 children aged 6 to 37 months attending the day care center, and 23 staff members. In July and December, a single nasopharyngeal swab was obtained from each child and staff member studied. The Ethics Committees in charge of health care for the day care center approved the research protocol. Signed informed consent was obtained for each child from a parent or guardian.

Nasopharyngeal swab specimens were collected by inserting a flexible swab (Culture Swab Transport System, Difco laboratories, Detroit, MI, USA) through the nares until the posterior pharynx was reached. The swab was then rotated for 1-2sec and withdrawn. Swabs were placed in transport medium (Stuart, modified) and carried to the laboratory to be processed on the same day.

Bacterial culture and identification

Swabs were streaked on chocolate agar plates to isolate *Hi*, and the plates were then incubated for 24 to 48 hours at 35°C in an atmosphere containing 5% CO_2. Colonies suspected of being *Haemophilus* were confirmed by Gram's staining and their requirement for V and X factors for growth. Species and biotypes were identified in each isolate as described by Kilian and Biberstein [16]. The latex-agglutination technique with antiserum was used to identify serotypes a to f (Difco).

Bacterial strains

The strains used were NCTC 7279 for wild type *Hi*b and *Escherichia coli* DH5α for plasmid propagation. Isolates were stored on a long-term basis at - 70 °C in supplemented brain heart infusion broth containing 80% glycerol (1:1).

DNA probes

Plasmid pKK3535 (11.8Kb) [17] was provided by Irma Rivera (Biomedical Institute, São Paulo University, Brazil). This plasmid contains the entire rRNA operon (5S, 16S, 23S) of the *E. coli* chromosome.

DNA preparation

High-molecular-weight chromosomal DNA was extracted from *Hi* isolates [18]. Plasmid DNA was isolated by the alkaline lysis method [19].

Southern analyses of genomic DNA

Genomic DNA (3 μg) was fully digested with *Hind*III (Bethesda Research Laboratories, Gaithersburg, MD, U.S.) according to manufacturer instructions, eletrophoresed on 0.7% agarose-Tris acetate gels, and transferred to nylon membranes. Nick translation and Southern hybridization were performed by standard techniques [19]. Ribotyping for 16S and 23S was done by a method described before [20] and using the pKK 3535 plasmid [17].

Results

Culture specimens were obtained from 38 children and 23 day care staff members who agreed to participate in the study. This subpopulation comprised 61% of the total number of children enrolled at the center and 88% of the day care staff. The nasopharyngeal cultures were positive for *Hi* in 28 of 37 children (75.6%) in July, and in 30 of 38 children (78.9%) in December. For the adult staff members, only 1 of 23 (4.35%) in July, and 1 of 23 (4.35%) in December were positive for *Haemophilus*.

A total of 60 isolates were biotyped and serotyped. The adult staff member isolate recovered in July was an *Hi* biotype II, and the isolate recovered in December was *H. paraphrohaemolyticus*. The *Hi* serotypes recovered from children in July were serotypes b (child 18) and e (child 21), but in December, only serotype b was found (children 12, 22, 23, 35 and 37) (Table 1). The capsule-type b strains were biotype I in July, and biotype I, II, and V in December (Table 1). Except for the *Hi* isolate with the above serotype and the recovered *H. parainfluenzae,* all other strains were NT*Hi* isolates.

In July, the predominant biotypes found among the *Hi* isolates were II (39%), V (25%) and I (21%). In December, the variability increased and only biotype III was found at a high percentage (30%), while other species such as *H. parainfluenzae* were recovered (Table 2).

The rRNA genes were visualized by probing with the radioactive labeled plasmid pKK3535 DNA. Digestion with *Hind* III restriction enzyme resulted in 10 main bands of 18, 8.8, 7.8, 5.8, 4.8, 3.7, 2.4, 2.1, 1.9 and 1.1 Kb. After *Hind* III cleavage, the strains could be differentiated into 40 ribotype patterns and grouped. Table 3 shows the variability of ribotypes from the isolates collected in July and December. Thirteen patterns were found with more than 1 isolate, and 27 other patterns were found only once and were designated variable. Among the 28 *Hi* isolates from the first collection (July), 19 (68%) showed a ribotype pattern present in more than 1 strain, ribotype I was recovered from different subgroups, and ribotype IV was recovered at highest incidence from the same subgroup. Only ribotype V was found in both July and December.

Discussion

Bacteria present in the nasopharynx of normal children reflect the strains currently circulating in the community and causing infections. Therefore, study of the prevalence of these bacteria can provide useful information.

With the exception of limited data available from studies of *Hi*b strains recovered in restricted settings in Brazil and Chile [21], little is known about the nature of strains cultured from patients in South America. Analysis of the available data indicates that South America does not differ significantly from other geographic regions [15, 22], and, until recently, the incidence and scope of *Hi*b diseases appear to be similar to those observed in the United States [23] and Europe [24].

The prevalence of nasopharyngeal *Hi* at the day care center investigated was quite high (77%) when compared with the nasopharyngeal frequency among Italian children [25], but was similar to colonization rates reported for American children [26]. The biotypes isolated, I, II and III, agree with those reported by others [27].

Spinola [28] found that children were usually colonized by a strain of NT*Hi* for several months, which was then substituted by a new strain, indicating that the

Table 1. Biotypes and serotypes of *Haemophilus influenzae* isolated from the nasopharynx of children at a day care center

Child	Group	Age in months	Doses of conjugate vacine	1st collection	2nd collection
1	A	6	0	Neg.	Neg.
2	A	6	1	Neg.	Neg.
3	B	9	0	I	VI
4	B	9	0	V	III
5	C	10	0	I	parainfluenzae I
6	C	10	0	V	Neg.
7	C	11	0	I	VI
8	C	11	0	Neg.	Neg.
9	C	11	0	Neg.	Neg.
10	C	12	0	I	Neg.
11	D	15	0	III	I
12	D	18	0	II	Vb
13	D	19	0	V	II
14	D	19	3	Neg.	I
15	D	20	1	III	II
16	E	19	3	II	Neg.
17	E	21	0	II	III
18	E	21	0	Ib	III
19	E	22	2	V	IV
20	F	23	0	II	II
21	F	23	0	IV e	V
22	F	23	0	II	Ib
23	F	23	2	n.c	Ib
24	G	29	0	II	III
25	G	29	0	I	V
26	G	31	0	I	V
27	G	31	1	II	parainfluenzae II
28	G	34	1	II	III
29	G	34	0	II	III
30	G	34	0	V	II
31	H	32	0	Neg.	Neg.
32	H	36	0	II	III
33	H	36	0	V	IV
34	H	36	1	Neg.	III
35	H	36	1	Neg.	IIb
36	H	37	0	II	I
37	H	37	1	V	IIb
38	H	37	1	Neg.	III

Different modules inside day care.

A,B,C,D,E,F,G,H: subgroups inside modules; I,II,III,IV,V,VI: biotypes of *Haemophilus*; b, e: serotypes of *Haemophilus influenzae*; Nc: not collected; Neg: without positive isolation.

Table 2. Percentage of various biotypes of *Haemophilus influenzae* and *Haemophilus parainfluenzae* isolated at the first and second collection from the nasopharynx of children at a day care center

Haemophilus	**1st coll. (n=28)**	**2nd coll. (n=30)**
influenzae biotype I	21	10
influenzae biotype Ib	4	7
influenzae biotype II	39	13
influenzae biotype IIb		7
influenzae biotype III	7	30
influenzae biotype IV		7
influenzae biotype IVe	4	
influenzae biotype V	25	10
influenzae biotype Vb		3
influenzae biotype VI		7
parainfluenzae biotype I		3
parainfluenzae biotype II		3

* Percentage of positive isolates.

Table 3. Distribution of ribotypes found with the pKK3535 probe after cleavage by *Hind*III

Group	A		B		C		D		E		F		G		H		Total of children	
Collection	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Similar Ribotype I			Δ				Δ		Δ				Δ				4	0
Similar Ribotype II				Δ								Δ					0	2
Similar Ribotype III					ΔΔ												2	0
Similar Ribotype IV					Δ		ΔΔΔ										4	0
Similar Ribotype V								Δ			Δ		Δ		Δ		2	1
Similar Ribotype VI									Δ								2	0
Similar Ribotype VII											Δ		Δ				2	0
Similar Ribotype VIII											Δ		ΔΔ				3	0
Similar Ribotype IX														ΔΔ			0	2
Similar Ribotype X													Δ			Δ	0	2
Similar Ribotype XI														Δ		Δ	0	2
Similar Ribotype XII																ΔΔ	0	2
Similar Ribotype XIII																Δ	0	1
Variable Ribotypes			Δ	Δ	Δ	ΔΔ		ΔΔΔΔ	ΔΔ	ΔΔΔ		ΔΔΔ	ΔΔ	ΔΔΔ	ΔΔΔ	ΔΔ	9	18
Total strains 1st col.	0		2		4		4		4		3		7		4		28	
Total strains 2nd col.		0		2		2		5		3		4		7		7		30

*A, B, C, D (infants- aged 6 to 20 months), E, F, G, H (toddlers- aged 19 to 37 months): subgroups of children at the day-care center; **I to XIII : ribotypes found more than once; *** variable ribotypes: ribotype found only once; Δ: one child.

loss and acquisition of strains was a common event. Our data confirm this observation because most of the children showed a different biotype strain in the first compared to the second collection (Table 1).

Hi is carried in the throats of most healthy adults and can be spread by droplet infection [29]. Surprisingly, we recovered only two *Hi* isolates from two different day care staff members (one in July, and one in December), even though we examined swabs from 23 adults each time. This was unexpected because high *Hi* carriage rates have been described in adults, especially NT*Hi* strains [30]. Neither strain recovered was similar to those recovered from the children (data not shown). An explanation for the small number of isolates recovered from the adults could be the sampling method. Coen, et al. [31], found that oropharyngeal cultures are more sensitive than nasopharyngeal cultures.

We also investigated the source of variation detected in traditional probe-based ribotyping patterns of NT*Hi*. Previous ribotyping studies of *Hi* showed that *Eco*RI or *Hind*III gave the most discriminating patterns [32]. Jordens and Leaves [33] reported that traditional 16S+ 23S rRNA remains the standard for epidemiological investigation of NT*Hi*. We found 40 different ribotypes, 13 of them recovered from more than 1 isolate, while 27 other ribotypes were found only once (Table 3). The results suggest a high level of strain shift between July and December.

Very young infants are protected against *Hi*b by passively-acquired maternal antibodies. However, after the first few months of life, the incidence of *Hi*b infection rises dramatically, with a peak incidence between 6 and 11 months of age. This incidence then falls slowly in parallel to the development of naturally occuring antibodies so that, by about 2 years of age, the incidence has fallen sharply and, by 5 years of age, the age-specific incidence begins to level off at a relatively constant low level [34].

Pharyngeal colonization by *Hib* is present in less than 5% of healthy preschool children who have not been exposed to a patient with systemic disease [35]. In contrast, carrier rates as high as 50% have been found in preschool children who attend day care centers in which one or more individuals with *Hi*b disease were present [36]. Limited information is available describing the natural history of *Hi*b colonization among children in day care in the absence of invasive disease. Turk [37] reported that 9 (11%) of 85 healthy preschool children at a day care nursery without disease were colonized by *Hi*b. We found almost the same frequency (12%) of *Hi*b, but with a difference; i.e., 3 children (25%) had already received one or two doses of vaccine and were considered adequately immunized against *Hi*b [15].

In Brazil, three vaccines were released for use in two-month-old infants; the Act-HIB (Pasteur-Mérieux, Lyon, France), the HibTITER (HbOC Wyeth-Lederle, Pearl River, NY, USA) and the PedvaxHIB (PRP-OMP Merck Sharp and Dohme, West Point, Pennsylvania, USA). Act-HIB and HibTITER should be administered at 2, 4, and 6 months, with a booster dose at 15 months, and the PedvaxHIB should be administered at 2 and 4 months with a booster dose at 12 months. For those vaccinated after age 12 months, a single dose of any vaccine should be administered [38]. The use of a booster dose is a controversial question and, in the United Kingdom and the Republic of Ireland, the booster dose is not administered [39]. Children < 1 year of age are considered adequately immunized against *Hi*b if, prior to testing, they have received 3 doses of vaccine (when immunization begins before age 6 months) or 2 doses (when immunization begins between 7 and 12 months of age). For those vaccinated after age 12 months, a single injection is considered satisfactory.

Children 23, 35 and 37 (Table 1) were considered to have been immunized against *Hi*b, but they still carried *Hi*b strains in their nasopharynx. The observation that *Hi*b is still circulating in the community is a cause of concern. If *Hi*b vaccination levels fall, existing *Hi*b clones could spread more widely and cause an increase in *Hi*b disease [40].

The incidence of *Hi*b disease in Brazil is difficult to evaluate [41], and only cases of meningitis could be adequadely evaluated. Still, the numbers obtained are confusing and are probably underestimated. Personal information obtained from the Regional Health Office

of Ribeirão Preto indicated that a small number of cases (less than 2/100 cases) of meningitis were caused by *Hi*b each year.

In many countries where *Hi*b conjugate vaccines have been introduced, there has been a dramatic reduction in the incidence of *Hi*b disease [3, 5, 42-45]. This reduction was often more marked than could be attributed to the direct effect of vaccination and included protection from disease in age groups not included in the vaccination program . This has been attributed to a herd protective effect achieved through a reduction in carriage [5]. The herd protective effect was not detected in the present study, probably because the population evaluated was small or because the time of observation was too short.

Day care provides a potential environment for high rates of acquisition of *Hi*b, even in the absence of invasive disease [46]. The rates of carriage found in the present study reinforce the necessity for constant monitoring to be sure that the national program of *Hi*b immunization introduced in August, 1999, has impacted the carriage as well as disease rates.

Acknowledgements

We thank Mrs. Ana Maria Mello, Director of the day care center, for permitting us to conduct the study and for obtaining informed consent from the parents of enrolled children. We are indebted to Mrs. Suzel Nogueira Neme and Mr. Paulo da Silva, Instituto Adolfo Lutz, for laboratory assistance, to Irma Rivera, Biomedical Institute, São Paulo University, for providing the plasmid pKK3535, and to Electra Green for reviewing the manuscript.

References

1. Turk D.C., Clinical importance of *Haemophilus influenzae*: 1981. In: Sell, S.H., Wright, P.F.eds. *Haemophilus influenzae*: Epidemiology, Immunology, and Prevention of Diseases. New York: Elsevier, **1982**.
2. Pittman M. Variation and type specificity in the bacterial species *Haemophilus influenzae*. J Exp Med **1931**;53:471-95.
3. van Alphen L., Spanjaard L., van Derende A., et al. Effect of nationwide vaccination of 3-month-old infants in the Netherlands with conjugate *Haemophilus influenzae* type b vaccine: high efficacy and lack of herd immunity. J Pediatr **1997**;131:869-73.
4. Liptak G.S., McConnochie K.M., Roghmann K.J., Panzer J.A. Decline of pediatric admissions with *Haemophilus influenzae* type b in New York State, 1982 through 1993: relation to immunizations. J Pediatr **1996**;130:923-30.
5. Adegbola A.A., Mulholland E.K., Secka O., Jaffar S., Greenwood, B.M. Vaccination with a *Haemophilus influenzae* type b conjugate vaccine reduces oropharyngeal carriage of *H. influenzae* type b among Gambian children. J Infect Dis **1998**;177:1758-61.
6. Klein J.O. Role of nontypeable *Haemophilus influenzae* in pediatric respiratory tract infection. Pediatr Infect Dis J **1997**;16:5-8.
7. Foxwell A.R., Kyd J.M., Cripps A.W. Nontypeable *Haemophilus influenzae* pathogenesis and prevention. Microbiol Mol Biol Rev **1998**;62:294-308.
8. Farley M.M, Stephens D.S., Brachaman P.S., et al. Invasive *Haemophilus influenzae* disease in adults: a prospective, population-based surveillance. Ann Inter Med **1992**;116:806-12.
9. Kuklinska D., Kilian M. Relative proportions of *Haemophilus influenzae* in the throat of healthy children and adults. Eur J Clin Microbiol **1984**;3:249-52.
10. Henderson F.W., Collier A.M., Sanyal M.A., et al. A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion . N Engl J Med **1982**; 306: 1377-83.
11. Trotier S., Stenberg K., Svanborg-Eden C. Turnover of non-typeable *Haemophilus influenzae* in the nasopharynges of healthy children. J Clin Microbiol **1989**;27:2175-9.
12. Forsgren J., Samuelson A., Ahlin A., et al. *Haemophilus influenzae* resides and multiplies intracellularly in human adenoid tissue as demonstrated by *in situ* hybridization and bacterial viability assay. Infect Immun **1994**;62:673-9.
13. Bresser P., van Alphen L., Habets F.J., et al. Persisting *Haemophilus influenzae* strains induce lower levels of interleukin-6 and interleukin-8 in H292 lung epithelial cells than nonpersisting strains. Eur Respir **1997**;10:2319-26.
14. Brunham R.C., Plummer F.A., Stephens R.S. Bacterial antigenic variation, host immune response, and pathogen-host coevolution. Infect Immun **1993**;61:2273-6.

15. Forleo-Neto E., de Oliveira C.F., Maluf E.M., et al. Decreased point prevalence of Haemophilus influenzae type b (Hib) oropharyngeal colonization by mass immunization of Brazilian children less than 5 years old with Hib Polyribosylribitol phosphate polysaccharide-tetanus toxoid conjugate vaccine in combination with diphtheria-tetanus toxoid-pertussis vaccine. J Infect Dis **1999**;180:1153-8.
16. Kilian M., Biberstein E.L. Genus *Haemophilus* In: Krieg, N.R., Holt, J.G. eds. Bergey's Manual of Systematic Bacteriology. Baltimore: Williams & Wilkins, **1984**.
17. Grimont F., Grimont P.A.D. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann Inst. Pasteur Microbiol. **1986**;137:165-75.
18. Pitcher D.G., Saunders N.A., Owen R.J. Rapid extraction of bacterial genomic DNA with guanidine thiocyanate. Lett Appl Microbiol **1989**;8:151-6.
19. Sambrook J., Fritsch E.F., Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, Laboratory Press, **1989**.
20. Irino K., Grimont F, Casin I., et al. rRNA gene restriction patterns of *Haemophilus influenzae* biogroup aegyptius strains associated with Brazilian Purpuric Fever. J Clin Microbiol **1988**;26:1535-8.
21. Musser J.M., Kapur V. Molecular population, genetics of *Haemophilus influenzae*. In: Ellis, R.W., Granoff, D.M. eds. Development and clinical uses of *Haemophilus* b vaccine.New York, Marcel Dekker, **1994**.
22. Peltola H. *Haemophilus influenzae* type b disease and vaccination in Latin America and the Caribbean. Pediatr Infect Dis J **1997**;16:780-5.
23. Ward J.I. Is *Haemophilus influenzae* type b disease preventable? JAMA **1985**;253:554-6.
24. Peltola H., Rod T.O., Jonsdottir K., et al. Life-threatening *Haemophilus influenzae* infections in Scandinavia: a five country analysis of the incidence and the main clinical and bacteriologic characteristics. Rev Infect Dis **1990**;12:708-15.
25. Principi N., Marchisio P., Schito G.C., et al. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Pediatr Infect Dis J **1999**;18:517-23.
26. Faden H., Duffy L., William A., et al. Epidemiology of nasopharyngeal colonization with nontypeable *Haemophilus influenzae* in the first 2 years of life. J Infect Dis **1995**;172:132-5.
27. Oberhofer F.R., Back A.E. Biotypes of *Haemophilus influenzae* encoutered in clinical laboratories. J Clin Microbiol **1979**;10:168-74.
28. Spinola S.M., Peacock J., Denny F.W., et al. Epidemiology of colonization by nontypeable *Haemophilus influenzae* in children: a longitudinal study. J Infect Dis **1986**;154:100-8.
29. Moxon E.R. *Haemophilus influenzae*. In: Mandel G.L., Bennet J.E., Dohn R. [eds.] Principles and practice of infectious diseases. 4th ed. New York, Churchill Livingstone, **1995**.
30. Sarangi J., Cartwright K., Stuart J., et al. Invasive *Haemophilus influenzae* diseases in adults. Immunol Infect **2000**;124:441-7.
31. Coen P.G., Heath P.T., Garnet G.P. The Hib immunization programme in the Oxford region: an analysis of the impact of vaccine administration on the incidence of disease. Epidemiol Infect **1999**;123:389-402.
32. Bruce K.D., Jordens J.Z. Characterization of noncapsulate *Haemopilus influenzae* by whole-cell polypeptide profiles, restriction endonuclease analysis and rRNA gene restriction patterns. J Clin Microbiol **1991**;29:291-6.
33. Jordens J.Z., Leaves N.I. Source of variation detected in ribotyping patterns of *Haemophilus influenzae*: comparison of traditional ribotyping, PCR-ribotyping and rDNA restrictrion analysis. J Med Microbiol **1997**;46:763-72.
34. Shapiro E.D., Ward J.L. The epidemiology and prevention of disease caused by *Haemophilus influenzae* type b. Epidemiol Rev **1991**;13:113-42.
35. Michaels R.H., Poziviak C.S., Stonebraker F.E., Norden C.W. Factors affecting pharyngeal *Haemophilus influenzae* type b colonization rates in children. J Clin Microbiol **1976**;4:413-7.
36. Granoff D.M., Gilsdorf J., Gessert C.E., Lowe L. *Haemophilus influenzae* type b in a day care center: Relationship of nasopharyngeal carriage to development of anticapsular antibody. Pediatrics **1980**;65:65-9.
37. Turk D.C. Nasopharyngeal carriage of *Haemophilus influenzae* type b. J Hyg **1963**;61:247-56.
38. Weckx L.Y., Carvalho E.S. Calendário vacinal: dinâmica e atualização. J Pediatria **1999**;75:149-54.
39. Heath P.T., Booy R., Griffiths H., et al. Clinical and immunological risk factors associated with *Haemophilus influenzae* type b conjugate vaccine failure in childhood. Clin Infect Dis **2000**;31:973-80.
40. Smith-Vaughan H.C., Sriprakash K.S., Leach A.J., et al. Low genetic diversity of *Haemophilus influenzae* type b compared to nonencapsulated *H. influenzae* in a population in which *H. influenzae* is highly endemic. Infect Immun **1998**;66:3403-9.
41. Bouskela M.A.L., Grisi S., Escobar A.M.U. Aspectos epidemiologicos da infecção por *Haemophilus influenzae* tipo b. Rev Panam Salud Publica **2000**;7:332-9.
42. Barbour M.L., Mayon-White R.T., Coles C., et al. The impact of conjugate vaccine on carriage of *Haemophilus influenzae* type b J Infect Dis **1995**;171:93-8.

43. Muhlemann K., Balz M., Aebi S., Schopfe K. Molecular characteristics of *Haemophilus influenzae* causing invasive disease during the period of vaccination in Switzerland: analysis of strains isolated between 1986 and 1993. J Clin Microbiol **1996**;34:560-3.
44. Tsolia M.N., Theodoridon M.N., Mostron G.J., et al. Epidemiology of invasive *Haemophilus influenzae* type b infections among children in Greece before the introduction of immunization. Scand J Infect Dis **1998**;30:165-8.
45. Scheifele D.W., Halperin S.A., Guasparini R., et al. Extended follow-up of antibody levels and antigen responsiveness after 2 *Haemophilus influenzae* type b conjugate vaccines. J Pediatr **1999**;135:240-5.
46. Murphy T.V., Granoff D., Chrane D.F., et al. Pharyngeal colonization with *Haemophilus influenzae* type b in children in a day care center without invasive disease.J Pediatr **1985**;106:712-6.