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Oropharyngeal Colonization of *Haemophilus influenzae* Type b and Serologic Response After Administration of Third Dose of Pentavalent Vaccine to 12-Month-Old Children in Karaj, Iran, 2016

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Abstract

Background: The administration of *Haemophilus influenzae* type b (*Hib*) conjugate vaccine led to a decrease of over 90% in the prevalence of severe *Hib* diseases in the countries with universal coverage vaccine. After addition of *Hib* vaccine to the national vaccination program and since no study has yet investigated this subject.
Objectives: The current study aimed at investigating the serologic response and assessing oropharyngeal colonization with *Hib* after the last dose of vaccine.
Methods: A total of 500 blood and oropharyngeal samples were collected from one-year-old children referred to Karaj health care centers, Iran. Demographic information and risk factors of the children were collected. Oropharyngeal and blood samples were transferred to the laboratory to determine antibody titer by the enzyme-linked immunosorbent assay (ELISA) technique, culture testing, and polymerase chain reaction (PCR).
Results: In the current study, 11.8% of children (95% confidence interval (CI): 8.97 - 14.63) had an anti-*Hib* IgG titer of $\geq$ 5 μg/mL. Geometric mean titer (GMT) of vaccine antibody was 6.92 μg/mL (95% CI: 6.76 - 7.08); 9% of oropharyngeal culture results were positive for *H. influaenzae* (non-type b) and 8.2% were confirmed by PCR. Prevalence of oropheryngeal *Hib* colonization was 0.02%. There was no significant correlation between the titer of *H. influaenzae* antibody and positive culture of *H. influaenzae* and the other studied variables ($P > 0.05$).
Conclusions: In Iran, similar to most countries, pentavalent vaccine in national vaccination program decreased the prevalence of *Hib* colonization. Prevalence of *Hib* colonization is an important factor in invasive diseases incidence. It is suggested that further studies asses the prevalence of invasive *Hib* diseases after national vaccination.

Keywords: Childhood, *Haemophilus influenzae* Type b, Vaccination

1. Background

Haemophilus influenzae, a pleomorphic Gram-negative microorganism, can remain as an asymptomatic carrier, but become invasive leading to pneumonia, septic arthritis, or meningitis (1). In 2000, the two leading causes of vaccine preventable diseases in children under five years old were *H. influenzae* type b (*Hib*) and *Streptococcus pneumonia*. *Hib* is the cause of three million serious disease cases and 386,000 deaths every year (the World Health Organization (WHO) report). The mean global case fatality rate (CFR) of *Hib* invasive disease is reported 43% (ranging 23% - 55%) and 44% (ranging 26% - 62%) in Eastern Mediterranean region (EMRO 2000) (2). The WHO recommended pneumococcal conjugate vaccine (PCV) and *Hib* conjugate vaccine (*Hib*CV) to be incorporated into routine vaccination in all countries, especially those with high children mortality rates (3).

In the developed countries, 92% of the people are vaccinated against *Hib* since 2003 (4-6). In 2008, 136 out of 194

member states of the WHO started *Hib* vaccination, when 203,000 deaths were attributed to *Hib* disease in the children under five years old (7). PCV and *Hib*CV played special roles in reducing children mortality rate by two-thirds from 1990 to 2015, after the implementation of the Millennium Development Goal (MDG) (8).

The incidence and severity of pneumonia and meningitis were reduced by *Hib* vaccination (9-13). *Hib* vaccination coverage increased in recent years. Since 2013, about 98% of the WHO member states and 52% of infants across the world received *Hib* vaccine through their immunization programs (14).

There are inconsistent results regarding serologic response to *Hib* vaccination (15, 16). The rate of nasopharyngeal *Hib* colonization is 1% -10% in different populations of regions without routine *Hib* vaccination. Vaccination may change the subpopulation of colonization in pharyngeal regions from capsulated to non-capsulated, and therefore, change the disease behavior (17). The *Hib* carriage prevalence was reported 7.6% by culture from oropharyngeal swabs in 1000 children from 2005 to 2006 in Tehran, Iran. The study suggested implementation of *Hib* vaccination in Iran (1). Indian pentavalent vaccine was introduced into Iran vaccination program in 18 November 2014 and was replaced with DTWP vaccine in routine vaccinations of two, four, and six months (18).

2. Objectives

After introduction of *Hib* vaccination in Iran, estimation of serologic response to *Hib* and pharyngeal colonization seems necessary; therefore, the current study aimed at estimating the post-vaccination polymerase chain reaction (PCR) and throat culture for *H. influenzae*, and investigating its serologic response in one-year-old children in Karaj, Iran.

3. Methods

The current cross sectional study was conducted on healthy children attending Karaj health care centers in Alborz province, Iran for routine vaccination. Based on the estimated prevalence of 17% colonization rate (19), and estimation of the prevalence (P) that is clinically worthwhile to detect (d) as 4%, the sample size was 500. The vaccine used from 2014 in Iran is Pentavac (DTP/HB/*Hib*) in which SiiHibPRO (*Haemophilus* type b conjugate vaccine (intraperitoneally; I.P.) is reconstructed with diphtheria, tetanus, pertussis, and hepatitis B vaccine adsorbed (SII Q-VAC) (DTP-HB vaccine), supplied by Serum Institute of India Ltd. (Serum Institute of India Vaccination Brochure). Inclusion criteria were being one year old (between 11 months and 0 day, and 11 months and 29 days), history of complete vaccination confirmed by vaccination card, and normal growth based on the growth chart. Exclusion criteria were lack of parents' consent to allow their child participation in the study, lack of child's cooperation with the study, history of congenital or acquired immunodeficiency, history of transfusion of blood and blood products, malignancy and consumption of immunomodulators, and receiving intravenous immunoglobulin (IVIG) three months before sampling.

Sampling was performed by multistage cluster method. Birth weight, weight, height, breast feeding status, duration of breast feeding, the number of siblings, parents' educational level, child maintenance status, history of parental cigarette smoking, and history of respiratory tract infections were recorded by a trained person. After obtaining informed consent from the parents, the oropharyngeal swab was obtained from tonsillar area and posterior throat, with a sterile cotton-tipped swab applicator. The swab was directly introduced into the oropharyngeal region and rotated for 1 - 2 seconds. All samples were cultured on chocolate agar and transferred to a referral laboratory. The culture media were incubated at 35°C - 37°C for 24 hours in a candle jar to produce CO_2. Bacteria were diagnosed based on colony morphology and Gram stain; polymorph Gram-negative microorganisms were confirmed by microscope. Colonies were transferred from chocolate agar to blood agar and Mueller-Hinton agar and factors needed for growth of *H. influenzae*, such as X and V factors, were added. After a further incubation for over 24 hours and detection of growth on plates, colonies were utilized for species isolation by PCR (1).

The DNA extraction method of bacterial nucleic acids was based on the DNA Mini Kit protocol (Qiagen, Hilden, Germany). DNA from bacteria, *Omp*, *Bex*, and *B.Omp* genes were included during PCR, to confirm species identification and differentiate true non-typable *H. influenzae* strains from genetically cap locus-positive strains, respectively. One primer set was used to detect encapsulated *Hib*. All primer sequences with their amplicon size are listed in Table 1. For routine screening, PCR was performed with Taq polymerase (New England BioLabs, Ipswich, MA) (20, 21).

Blood sampling was performed on each child once. Blood samples were obtained from wrist and forearm veins by educated health workers, considering hand hygiene with an antiseptic other than chlorhexidine. Two milliliters of blood was drawn slowly and steadily to prevent hemolysis. Blood samples were maintained in a sterile, capped test tube and transferred to the main laboratory up to four hours after sampling for serum separation. Serum samples were stored at -70°C until the enzyme-linked immunosorbent assay (ELISA) was performed.

Table 1. Primers Sequences

Primer		Primer Sequence (5' to 3')	Amplicon Size (bp)	Reference
Omp6			351	(20)
	F	AACTTTTGGCGGTTACTCTG		
	R	CTAACACTGCACGACGGTTT		
BexA			343	(21)
	F	CGTTTGTATGATGTTGATCCAGAC		
	R	TGTCCATGTCTTCAAAATGATG		
B			480	(21)
	F	GCGAAAGTGAACTCTTATCTCTC		
	R	GCTTACGCTTCTATCTCGGTGAA		

Antipolyribosyl ribitol-phosphate (PRP) exists in the capsule of the bacteria measured by ELISA [IBL international kit, Germany (catalogue reference No.: RE56351)]. The patients' serum specific antibodies were banded to antigen coated wells and detected by a secondary enzyme conjugated antibody (E-Ab) (enzyme antibody) specific for human IgG. The color developed after the substrate reaction with Ag-Ab complex was detected. The results of optic density of samples were determined directly according to the standard curve. The results were reported as IU/mL; the lowest detectable level was 1 IU/mL.

The study protocol was approved by Research Council and Ethics Committee of Karaj University, and the permission of health officials was also obtained to enter the studied health centers (ethical approval code: Abzums.rec.1394.70). After explanation of the study purposes to the parents, all individuals that allowed the participation of their children in the study signed the informed consent forms, and then sampling was performed on their children. Quantitative variables were expressed as mean (standard deviation (SD)). The significance of difference between the studied variables and dependent qualitative and quantitative ones were investigated using both logistic and linear regression analyses. The data were analyzed at 95% confidence interval (CI) using logistic regression and $P < 0.05$ was considered the level of significance. Univariate analyses were included in a multivariate model to identify independent factors associated with carriage of *H. influenzae*.

4. Results

A total of 500 children were enrolled in the study, 255 were female (51%) and 119 passive smoker (23.8%); 113 (31%) mothers and 115 (23%) fathers had academic education; seven (1.4%) cases received kindergarten care, 78 (15%) were exclusively breastfed, 364 (72%) had a history of frequent upper respiratory tract infection (URTI), 462 (84.4%) had a history of breastfeeding in the first six months of age, 432 (86.4%) had less than two siblings. GMT of antibody response was 6.92 (95% CI: 6.76 - 7.08). IgG antibody in 59 children (11.8%) was equal or higher than 5 μg/mL (95% CI: 8.97 - 14.63) and antibody level in 441 cases (88.2%) was less than 5 μg/mL (95% CI: 85.37 - 91.03) (Table 2). Of the 500 cases, 45 (9%) (95% CI: 6.49 - 11.51) had positive culture for *H. influenzae*, 41 (8.2%) of which (95% CI: 5.8 - 10.6%) were confirmed by PCR. Protective level of antibody and culture results had no significant difference with respect to weight, birth weight, height, breastfeeding duration, gender, parents' educational level, maintenance status, parental smoking, exclusive breastfeeding, and frequent URTI analyzed by multivariate analysis (Tables 3 and 4). Typing was performed on PCR positive cases, showing that all cases were of nontypable and one case was positive for *Hib* (0.02%).

Table 2. Anti-*Haemophilus influenzae* Titer (IgG) and the Result of Nasopharngeal Culture in One-Year-Old Children

Variables, Titer	Culture		Total
	Negative	Positive	
***Hib* IgG, μg/mL**			
≤ 0.15	6	1	7[a]
0.15 - 1	190	18	208
1 - 5	206	21	227
> 5	53	5	58
Total	455	45	500

[a] Only one of 45 culture positive cases was positive with PCR for *Haemophilus influenzae* type b, and their serologic titer was 0.61 μg/mL.

5. Discussion

Oropharyngeal colonization with *Hib* may be a cause of bacteremia and invasive diseases. Increased antibody may occur at two years of age. Recurrent episodes of colonization may increase serum anti-PRP level (22). The rate of nasophayngeal colonization is affected by *Hib* vaccination. This effect may change the type and distribution of subtypes in vaccinated children by decreasing colonization of *Hib* suggested by many studies across the world. Before vaccination program, the prevalence of *H. influenzae* carriage in Thailand was 44.2% (23), in France 40.9% (24) and in Poland 49.9% (25). Nasopharyngeal colonization with *H. influenzae* in Scandinavian children in 1995 decreased from 13% in under seven-year-old children before mass vaccination to 6% in 7- to 14-year-old children and 3% in the ones over 16 years old (26). After the mass vaccination, nasopharyngeal colonization was 45% in French children aged 0 - 24

Table 3. Variables and Protective Anti-*Haemophilus influenzae* Titer in One-Year-Old Children

Variables	Not Protective < 5	Protective ≥ 5	Total	P Value
Weight, g[a]	9600 (1132)	9.74 (875.08)	-	0.35
Height, cm[a]	75.99 (3.55)	76.46 (3.22)	-	0.34
Breastfeeding duration, min[a]	10.25 (3.74)	10.70 (3.06)	-	0.37
Gender[b]				0.25
Male	212 (86.5)	33 (13.5)	245	
Female	229 (89.8)	26 (10.2)	255	
Siblings number[b]				0.99
< 2	381 (88.2)	51 (11.8)	432	
≥ 2	60 (88.2)	8 (11.8)	68	
Mother education[b]				0.37
Non academic	344 (88.9)	43 (11.1)	241	
Academic	97 (85.8)	16 (14.2)	113	
Father education[b]				0.88
Non academic	340 (88.3)	45 (11.7)	385	
Academic	101 (87.8)	14 (12.2)	115	
Maintenance status[b]				0.15
House	437 (88.6)	56 (11.4)	493	
Kindergarten	5 (71.4)	2 (28.6)	7	
Parent smoking[b]				0.75
Yes	104 (87.4)	15 (12.6)	119	
No	337 (88.5)	44 (11.5)	381	
Exclusive breastfeeding[b]				0.4
Yes	370 (87.7)	52 (12.3)	422	
No	71 (91.0)	7 (9.0)	78	
Brestfeeding[b]				0.8
Yes	407 (88.1)	55 (11.9)	462	
No	34 (89.5)	4 (10.5)	38	
Frequent upper respiratory tract infection[b]				0.23
Yes	124 (91.2)	12 (8.8)	136	
No	318 (87.4)	46 (12.6)	364	
Birthweight, g[b]				0.44
≤ 2500	35 (94.6)	2 (5.4)	37	
2500 - 4000	397 (87.6)	56 (12.4)	453	
≥ 4000	9 (90.0)	1 (10.0)	10	

[a] Values are expressed as mean (SD).
[b] Values are expressed as mean (%).

months (1993), and none of them were of serotype b (27). After *Hib* vaccination with coverage of > 90% in Brazilian children in 2002, nasopharyngeal coverage of *Hib* in 2006 was 1% for type b (28). After beginning mass *Hib* vaccination since 2001 in Kenya, *Hib* colonization under five-year-old children was 1.7% and coryza was the risk factor for its colonization (2007) (29). A study in Brazil on 6- to 24-month-old children reported *Hib* point prevalence of oropharyngeal colonization before vaccination as 1.2% vs. 4.8% after vaccination (30). Nasopharyngeal colonization in Iranian

Table 4. Variables and *Haemophilus influenzae* Culture Results in One-Year-Old Children

Variables	Negative	Positive	Total	P Value
Weight, g[a]	9604 (1108)	9759 (1077)	-	0.38
Height, cm[a]	76.007 (3.50)	76.53 (3.62)	-	0.35
Gender[b]				0.76
Male	224 (91.4)	21 (8.6)	245	
Female	235 (92.2)	20 (7.8)	255	
Siblings number[b]				0.22
< 2	394 (91.2)	38 (8.8)	432	
≥ 2	65 (95.6)	3 (4.4)	68	
Mother education[b]				0.49
Non academic	357 (92.2)	30 (7.8)	384	
Academic	102 (90.3)	11 (9.7)	113	
Father education[b]				0.54
Non academic	355 (92.2)	30 (7.8)	385	
Academic	104 (90.4)	11 (9.6)	115	
Maintenance status[b]				0.42
House	452 (91.7)	41 (8.3)	493	
Kindergarten	7 (100.0)	0	7	
Parent smoking[b]				0.29
Yes	112 (94.1)	7 (5.9)	119	
No	347 (91.1)	34 (8.9)	381	
Exclusive breastfeeding[b]				0.85
Yes	387 (91.7)	35 (8.3)	422	
No	72 (92.3)	6 (7.7)	78	
Breast feeding[b]				0.05
Yes	421 (91.1)	41 (8.9)	462	
No	38 (100.0)	0	38	
Frequent URI[b]				0.75
Yes	124 (91.2)	12 (8.8)	136	
No	335 (92.0)	29 (8.0)	364	
Birthweight, g[b]				0.38
≤ 2500	34 (91.1)	3 (8.1)	37	
2500 - 4000	417 (92.1)	36 (7.9)	453	
≥ 4000	8 (80.0)	2 (20.0)	10	

[a] Values are expressed as mean (SD).
[b] Values are expressed as mean (%).

children in 2011 was investigated by Mousavi on one- to six-year-old children 17% of whom were positive for *Hib* (19). Jalali et al., reported nasopharyngeal colonization with *Hib* in 25- to 48-month-old children attending day care centers in Tehran 33%, and 28% were confirmed by PCR (31). These studies did not address *Hib* colonization. In a study conducted on 1000 oropharyngeal cultures obtained from 25 day care centers (2005 - 2006) in Tehran, the prevalence of *Hib* carriage was 7.6% (1). This result was an important estimation of *Hib* colonization in Iranian children. In comparison with Karimi et al.'s (1) results before mass *Hib* vaccination in Iran, the current study results indicated that

oropharyngeal colonization decreased by 0.02% after mass vaccination.

The current study also highlighted on antibody response to *H. influenzae* and its relationship with oropharyngeal colonization. Due to higher sensitivity of oropharyngeal colonization, this method was preferred to nasopharyngeal colonization to obtain the organism (32).

Antibody-mediated response prevents pharyngeal colonization, confirmed by a study in BALB/c mice whose natural serum immunoglobulin could exhibit complement-dependent bactericidal activity by binding to bacterial surface (33). In a study conducted on the *Hib* carriers in infant rats, human secretory anti-*Hib* PS IgA inhibited nasopharyngeal colonization by inhibiting the mucosal growth and adherence (34).

Anti-*Hib* capsular polysaccharide antibodies of $\geq$ 0.15 μg/mL and $\geq$ 1 μg/mL were used to confirm short- and long-term protection against invasive disease. The relationship between anti-capsular polysaccharide and protection against immunization was studied in Dominican Republic in 1999. The prevalence of *Hib* colonization was significantly lower in vaccinated infants after administration of three doses at two, four, and six months than in the unvaccinated group (0.9% vs. 2.3%), which was directly related to anti-*Hib* CPS $\geq$ 5 μg/mL concentrations. Results showed that serum antibody level of $\geq$ 5 μg/mL could prevent pharyngeal colonization in vaccinated children (35). The current study used the $\geq$ 5 μg/mL cut-off point to investigate antibody level to prevent pharyngeal colonization in Iranian children. The results revealed that of the 500 vaccinated children, 58 had such antibody level. The important point about the current study was that only one child colonized with *Hib* in spite of $\leq$ 5 μg/mL antibody level in 227 out of 500 cases.

These results showed that there may be other mechanisms, promoted by vaccination, which may contribute to decreasing the rate of *Hib* colonization in Iranian vaccinated children.

Since *Hib* vaccination is becoming widespread, an increase in resistant and virulent non-typable *H. influenzae* may occur and the importance of its pathogenicity may increase, a new problem, namely, decrease in *Hib* and increase in non-typable *H. influenzae*, may be faced in the future. In the current study, approximately 10% of children were colonized with *H. influenzae* and only one case was of type b.

5.1. Conclusions

Results of the current study showed that in spite of low antibody level in approximately 50% of one-year-old Iranian children, nasopharyngeal colonization was observed in one child. This indicated that *Hib* vaccine, as a constituent of pentavalent vaccine successfully reduced *Hib* nasopharyngeal colonization. It is recommended that future studies investigate colonization and antibody status in Iranian children.

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Footnotes

Conflict of Interests: The authors declared no conflict of interest.

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