J Med Microbiol Infec Dis, 2014, 2 (1): 23-27

Original Article

Carriage Rate of Nasopharyngeal *Haemophilus Influenzae* among Children under 6 Years Old in Tehran, Iran

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Received Nov 2, 2013; accepted Dec 11, 2013

Introduction: *Haemophilus influenzae* is a gram-negative bacterium causing a variety of respiratory infections in developing countries, especially in children. Nasopharynx carriers of *H. influenzae* are the prominent source and transitional vectors of invasive diseases. As very limited information on *H. influenzae* carriage rate in Iran was available, an evaluation on prevalence of this bacterium in children ≤6 years old seems crucial. **Methods:** Totally 533 mucus samples were collected using nasopharyngeal swabs from children ≤6 years old who lived in 4 nursery centers in Tehran or refereed to the Children's Medical Center of Tehran, Iran, from August 2011 to October 2012. The samples were transported in Stuart transport medium to the Microbiology Laboratory of Pasteur Institute Tehran, Iran, and were cultured on chocolate agar containing bacitracin antibiotic. The initial diagnosis for detection of *H. influenzae* was performed by standard biochemical tests, and confirmation was achieved by PCR assay targeting outer membrane protein (*omp*) *P6* gene. **Results:** Based on primary cultures and biochemical tests, out of 533 samples, 182 (33%) showed to be *H. influenzae* positive, but PCR assay confirmed presence of *H. influenzae* in 153 (28%) isolates; 56 (37%) belonged to girls and 97 (63%) to boys. The prevalence of *H. influenzae* in three different age groups: ≤24, 25-48, and 49-72 month-old children were 31 (20%), 69 (45%), and 53 (35%), respectively. **Conclusion**: Our results showed a high rate of *H. influenzae* carriers among children ≤6 years old, which is similar to those of other unvaccinated countries. *H. influenzae* carriage rate was associated to age and respiratory infection diseases. The children aged 25-48 months showed a higher rate and the rate reduced with increase in age. Further investigation including molecular studies is required to obtain the carriage rate throughout the country. *J Med Microbiol Infec Dis, 2014, 2 (1): 23-27.*

Keywords: *Haemophilus influenzae,* Polymerase Chain Reaction, Nasopharynx, Iran.

INTRODUCTION

According to World Health Organization (WHO), infectious diseases contribute to death of many children worldwide, among which respiratory infections are prominent. Middle East is one of the high risk area for respiratory infections of children in the world [1-4]. The nasopharynx is a major reservoir area for upper respiratory pathogens and antibiotic-resistant bacteria in children. A variety of gram negative and gram positive bacteria such as causing agents of meningitis colonize in this area. The most common pathogens in nasopharynx are *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitides* [5, 6].

The main reason of colonization in the upper respiratory tract is *H. influenzae* (Hi). In the other words, *H. influenzae* is one of the most important human respiratory bacterium, which causes serious infections such as meni-ngitis, pneumonia, epiglottis, septicemia, sinusitis, and otitis media especially among children aged ≤6 years [2].

H. influenzae is a gram-negative, coccobacillary, facultatively anaerobic bacterium belonging to the Pasteurellaceae family. *H. influenzae* strains are divided into encapsulated, and noncapsulated strains, which are responsible for a wide range of localized and invasive infections. The invasive infections, *e.g.,* life threatening meningitis are caused by encapsulated *H. influenzae* type b (Hib). Nonecapsulated *H. influenzae* strains or nontype-able *H. influenzae* (NTHi) strains are associated with noninvasive infections and are commonly isolated from half of the middle ear infections with acute otitis media. Most *H. influenzae* strains are opportunistic pathogens; they usually exist in their host without causing any problem, but can cause serious infections when other factors (like viral infections, allergies, etc.) reduce immune system and create an opportunity for progression of the infection [1, 2, 7-11].

Effective vaccines for prevention of infections caused by type b of this bacterium have been available since 1990s, and are recommended for children under age 6 years and asplenic patients.

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The WHO recommends a pentavalent vaccine, a combination of vaccines against diphtheria, tetanus, pertussis, hepatitis B, and Hib. The Hib vaccine has been used for over 10 years in USA [1]. Before vaccination, the incidence of Hib meningitis was 11-50 in 100000 in children aged ≤4 in developed countries.

In industrialized countries, Hib infections affected more than 80% of ≤6 years old children; however, after vaccination this rate reduced to less than 1%.

About 92% of populations in developed countries have been vaccinated against Hib since 2003, whereas this rate is 42% for developing countries, and only 8% for under developed countries [3, 4, 7].

At the present, Iran is among the countries, which do not apply any vaccination against this bacterium, and the exact prevalence rate of this bacterium before application of any vaccination program is not available in this country. The aim of the current study was to determine the prevalence of *H. influenzae* colonization among children aged ≤6 years in Tehran, and to find the carriers prevalence of *H. influenzae* before application of a vaccination program. Outer membrane protein (OMP) P6 is one of *H. influenzae* lipoprotein. The *ompP6* gene that encodes this protein is highly conserved among all strains making it a reliable marker for *H. influenzae* diagnosis. We chose to study *ompP6* gene, because it is present in both encapsulated and noncapsulated strains [1, 3, 10, 12-15].

Materials and Methods

Study design and population. Totally 533 mucus samples were collected using nasopharyngeal swabs from children ≤6 years old who lived in 4 nursery centers (Ameneh, Shobeir, Torkamani and Roghaye) in Tehran, or refereed to the Children's Medical Center of Tehran, Iran, from August 2011 to October 2012. (The calculated study population size was based on the prevalence of 10%, α=0.05 at a confidence level of 95%). The data for each participant (name, age, sex, antibiotic usage, fever, vaccination status, respiratory infections, and day care center) was recorded in separate questioners. We obtained the written informed consent of the nurseries' officials or children's parents before sampling; they declared their willingness to allow the children's data to be used anonymously for research purposes.

Nasopharyngeal sampling. The nasopharyngeal swab was taken from the posterior throat and tonsil area of nasopharynx, using a commercial rigid cotton-tipped swab applicator which was introduced directly until resistance was felt. After sampling, swab applicators were placed into tubes containing Stuart transport medium. All the samples were transported immediately within 3 h to the Microbiology Center of Pasteur Institute, Tehran, Iran.

Bacteriology. The samples were cultured on chocolate agar plates supplemented with 260 µg⁄ml bacitracin. All plates were incubated aerobically at 35°C for 16 to 24 h in a 5% CO2-enriched atmosphere. Identification of *H. influenzae* was based on morphology of colonies (small, gray, and watery) and observation of gram negative cocobacills with positive catalase, oxidase, urea and endol tests.

Polymerase Chain Reaction. DNA extraction was done by boiling method as described by others [16]. Twenty four hours *H. influenza* colonies were suspended in 30 µl of sterile water, boiled for 7 min at 100°C and then centrifuged at 12000 for 3 min. Finally, 30 µl of supernatant was recovered and stored at 20°C. The primers used for detection of *H. influenzae* were F 5'- AACTTTTGGCGGTTACTCTG-3' and R 5'-CTAACACTGCACGACGGTTT-3' [19]. PCR was performed as described By Saiki *et al.* [17]. The DNAs from *H. influenzae* ATCC9007 and *Escherichia coli* ATCC 27117 were used as positive and negative control strains, respectively.

Amplification was performed in a Thermal Cycler (Eppendorf Mastercycler 5330; Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) programmed for 40 cycles, each consisted denaturation at 95°C for 45 s, annealing at 59°C for 30 s extension at 72°C for 45 s, with a final extension at 72°C for 6 min. The PCR products were visualized by electrophoresis in 1% agarose gel, stained with ethidium bromide and examined under ultraviolet illumination.

Statistical Analysis. Chi-square and Fisher exact tests were used for analysis of categorical data. Analyses were done using Sigma Stat for Windows version 2.03 (SPSS, Chicago, IL). A *p value* less than 0.05 was accepted as statistically significant.

RESULTS

This study comprised 553 samples; 218 (39%) belonged to girls and 335 (61%) to boys. The children were categorized by age into 3 age groups of ≤24, 25-48, and 49-72 months. According to data, 179 (32%) were in age group ≤24, 190 (34%) in age group 25-48, and 184 (33%) in age group 49-72 months.

Based on primary cultures and biochemical tests, 182 (33%) samples showed to be *H. influenzae* positive, but PCR assay confirmed presence of *H. influenzae* in 153 (28%) isolates (Table1) (Figure 1).

As table 2 indicates, There was a significant correlation between the infection of *H. influenzae* and age (*P value*=0.023), antibiotic consumption (*P value*=0.0012), respiratory diseases (*P value*=0.0025), cold symptom (*P value*=0.001), and the number of children, under 6 years old, living together in one room (*P value*=0.001).

Table 1. Prevalence of *H. influenzae* in children ≤6 years by routine culture and biochemical tests, and its confirmation by PCR using *ompP6* gene

Total Swabs No. (%)	Detection of *H. influenzae* by culture and biochemical tests No. (%)	confirmation of *H. influenzae* by PCR No. (%)	Coefficient No. (%)
553 (100%)	182 (33%)	153 (28%)	29 (5%)

There was a strong relationship between living in populated places and *H. influenzae* infection (*P value*=0.023). The highest frequency of *H. influenzae* infection was found in 25-48 months children, and this rate reduced with age. Ninety four (61%) of all *H. influenzae* carriers had used antibiotic within the past 10 d before sampling. In addition, 122 (80%) of carriers had cold symptoms. Seventy nine (52%) had a history of respiratory illness before sampling, and 74 (48%) of cases had not any respiratory diseases. There was a significant correlation between the prevalence of *H. influenzae* infection and number of children sharing the same room; as our data showed 103 carrier children (67%) shared a single room. Gender (*P value*=0.46) and fever (*P value*=0.782) were not significantly associated with *H. influenzae* carriage rate. None of children had received *H. influenzae* vaccine.

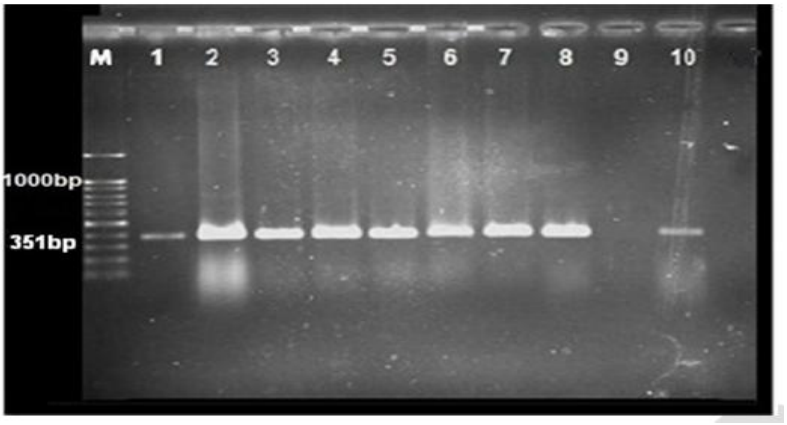

Fig. 1: Detection of *H. influenzae* DNA by amplification of 351 bp *ompP6* gene; Lane M, DNA ladder (1000 bp); lane 1-8, *H. influenza;* lane 9, negative control; lane 10, positive control.

Table 2. Characteristic of *H. influenzae* carriers and the associated factors

Variables		Positive No.	%	P value
gender	Boy	97	63	
	Girl	56	37	0.46
Age [months]	0-24	31	20	
	25-48	69	45	0.023
	49-72	53	35	
Antibiotic treated	Yes	94	61	
	No	52	39	0.0012
Fever	Yes	40	26	
	No	113	74	0.782
Respiratory disease	Yes	79	52	
	No	74	48	0.0025
Cold symptom	Yes	122	80	
	No	31	20	0.001
Attending kindergarten /Day care center	Yes	120	78	
	No	33	22	0.001
Number of households	<4	30	20	
	≥4	123	80	0.001

DISCUSSION

H. Influenzae is one of the most important causes of meningitis and pneumonia among children under 6 years old in developing countries. Before development of Hib conjugate vaccines, *H. influenzae* type b was responsible for more than 95% of all invasive *H. influenzae* infections worldwide; Hib still remains as a leading cause of meningitis among unvaccinated individuals in developing countries [18].

Infection in unprotected children can cause high morbidity and mortality. According to WHO, *H. influenzae* is responsible for more than 3 million cases of meningitis and 380,000 to 700,000 pneumonia annually worldwide [8, 9, 12, 19].

In this investigation, the prevalence of *H. influenza* among children ≤6 years old was 28%, which is in agreement with the results of similar studies in unvaccinated countries, which reported the carrier rates ranging from 12% to 72%. The first study in Iran conducted in 2004 in Imam Khomeini Hospital, Ahwaz, reported a prevalence of 8.23% for *H. influenzae* carriers [20]. In Mashhad, in 2007 this prevalence was 10.7% [19]. The higher rate (28%) of *H. influenzae* carriers in recent years may be due to accurate test for identifying this bacterium or lack of hygiene in the studied centers [20, 21].

According to a study in Thailand in 2010, the prevalence of carriers among children ≤6 years old was 44.4% [22]. The prevalence of carriers in children aged ≤6

in France [22] was 40.9%. Other studies in Poland [23] and Turkey [24] demonstrated carriage rates of 7.6% and 22.8% in children under 6 years old, respectively. All of these studies were done before vaccination program. The prevalence of *H. influenza* carriers in Iran is comparable with those in Thailand, France, Poland, and Turkey as vaccination against Hib is not a routine immunization program in these countries [11, 25, 26].

One of the most important findings of this study was the relationship between carriers and antibiotic consumption. Out of 153 isolates, 94 (61%) belonged to individuals who had consumed antibiotics orally (ampicillin and amoxicillin). This may be due to, bacterial resistance, time or dose of consumed antibiotic and/or other factors. In our survey the carriers rate was not affected by gender, this result is in accordance with those of Anderson *et al.* [27] who reported that men and women are equally affected by this bacterium. In this study 553 nasopharyngeal swabs were collected from children aged ≤6 years (39% girls and 61% boys). During the study, 182 (33%) *H. influenzae* strains were isolated using cultural and routine biochemical tests, but only 153 (28%) of the total samples were confirmed as *H. influenzae* by PCR assay. This result is in agreement with Van Ketel *et al*. [28] study who reported that PCR by targeting *p6* gene was a highly sensitive method for *H. influenzae* diagnosis than other methods of bacterial detection such as bacterial culture [13, 21, 29].

Our data revealed that bacterial colonization was associated to age, as 20%, 45%, and 35%, of *H. influenzae* isolates were obtained from ≤24, 25-48 and 49-72 months children, respectively. Therefore, the carriage rate was initially increased with age and then decreased. Clinical symptoms were associated with nasopharyngeal carriers of *H. influenzae*. A similar study by Anderson *et al.* [27] indicated that *H. influenzae* was the most prevalent causing agent of upper respiratory tract infections. Also, in our evaluation there was a strong relationship between upper respiratory infections and *H. influenzae* carriers [4, 5, 19, 21, 29].

As the prevalence of *H. influenzae* carriers has increased in recent years, quick and sensitive diagnosis method seems necessary. PCR assay may be one of the most useful and accurate techniques. The gene coding outer membrane protein P6 (OMP P6), which is conserved among all *H. influenzae* strains can be used as reliable tool for detection of *H. influenza* infection. In our study, culture result was positive for 33% of all samples, but PCR confirm 28% of them, revealing 5% false positivity for culture method in total samples [30-33].

Furthermore since the human is the only reservoir of *H. influenzae* and colonization has a relevant role in the transmission cycle of bacterial invasive diseases, a vaccination program against this bacterium seems to be essential for children under 6 years old specially when attending day care centers.

ACKNOWLEDGMENTS

We are indebted to Dr. Seyed Davar Siadat, for his kind discussion. We would also like to thank all staff of Children's Medical Center Hospital, The State Welfare Organization of Iran and the volunteer children and their families for participating in this study, and Dr. Anis Jafari for critical review of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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