Rapid and direct molecular detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* isolated in oropharynx and nasal cavity of children

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**Abstract**

There is a direct correlation between asymptomatic oropharyngeal colonization by pathogenic bacteria and the prevalence of paediatric respiratory infections. *Streptococcus pneumoniae* and *Haemophilus influenzae* are common cause of serious bacterial infections such as meningitis in children. This study aimed to monitor healthy children to detect asymptomatic carriers of *S. pneumoniae* and *H. influenzae*. In the present cross-sectional study, real-time PCR assay was developed and evaluated in comparison with culture for direct detection of *S. pneumoniae* and *H. influenzae* in 123 oropharynx and nasal cavity specimens from healthy children in Ilam, Iran. In addition, virulence factor (*ply* and *hpd*) and iron uptake (*tbpA* and *piuA*) genes were evaluated by PCR. Our results demonstrated that among all isolates only 14 *S. pneumoniae* and eight *H. influenzae* were identified by phenotypic methods, whereas 37 and 21 *S. pneumoniae* and *H. influenzae* were identified, respectively, by real-time PCR assay. All *S. pneumoniae* and *H. influenzae* isolates possessed *tbpA* and *piuA* genes. Also, 81% (30/37) of *S. pneumoniae* isolates were positive for *ply* gene, which encoded pneumolysin, as well as 90% (19/21) of *H. influenzae* isolates were positive for *hpd* gene. Simultaneous colonization of *S. pneumoniae* and *H. influenzae* could indicate the importance of monitoring of healthy children to identification of carriers.

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**Keywords:** Detection, *Haemophilus influenzae*, iron uptake, real-time PCR assay, *Streptococcus pneumoniae*, virulence factors

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The summary of demographic characteristics of healthy children in 37 Streptococcus pneumoniae and 21 Haemophilus influenzae isolates

| Characteristics                       | Streptococcus pneumoniae n (%) | Haemophilus influenzae n (%) |
|---------------------------------------|-------------------------------|-----------------------------|
| Sex                                    |                               |                             |
| Male                                  | 19 (31.7)                     | 11 (18.3)                   |
| Female                                | 18 (28.6)                     | 10 (15.9)                   |
| Age groups (years)                    |                               |                             |
| 2–3                                   | 19 (31.55)                    | 11 (52.4)                   |
| 4–5                                   | 4 (10.8)                      | 5 (23.8)                    |
| 6–11                                  | 2 (8.1)                       | 2 (9.5)                     |
| Delivery method                       |                               |                             |
| Caesarean section                     | 30 (54)                       | 13 (62)                     |
| Vaginal                               | 17 (46)                       | 8 (38)                      |
| Feeding children in the first 2 years of life |                       |                             |
| Breastfeeding                         | 30 (81)                       | 19 (90.5)                   |
| Formula feeding                       | 1 (2.7)                       | 0 (0)                       |
| Both                                  | 6 (16.3)                      | 2 (9.5)                     |
| Chronic respiratory diseases          | 3 (81)                        | 1 (4.8)                     |
| History of hospitalization            | 10 (27)                       | 5 (23.8)                    |
| Sampling                              | 16 (51.55)                    | 11 (52.4)                   |
| Winter                                | 4 (10.8)                      | 5 (23.8)                    |

Methods

Study design and participants

The present cross-sectional study was conducted from March 2014 to March 2015. One hundred and twenty-three throat and nasal swab samples were collected from children aged between 2 and 6 years in several kindergartens of Ilam, Iran. A questionnaire was completed by the parents that included: age, sex, history of hospitalization, history of chronic respiratory diseases (asthma, allergies), method of delivery (caesarean section or vaginal delivery), number of family members, smoking by other family members, feeding of infants in the first 2 years of life (breastfeeding or formula feeding or both), history of vaccination with conjugated pneumococcal-Haemophilus influenzae b, and Neisseria meningitidis vaccines. Healthy children without any symptoms of respiratory diseases and that had not received antibiotics in the last month matched the inclusion criteria in the present study. This study has been approved by the ethics committees of the Ilam University of Medical Sciences.

Sample collection, processing and isolation

The posterior oropharynx and nasal samples were collected by trained field workers using sterile dacron-tipped swabs (BD™ BBL; Becton Dickinson, Franklin Lakes, NJ, USA) and was immediately transported to skimmed milk, tryptone, glucose and glycerin (STGG) transport medium (Merck, Darmstadt, Germany) in appropriate conditions. The demographic characteristics of healthy children in 37 S. pneumoniae and 21 H. influenzae isolates are summarized in Table 1.

To increase the isolation of H. influenzae, 200 μL of inoculated STGG was added to brain–heart infusion broth, which contains 100 mg/L haemin and 2 mg/L NAD. Thereafter, it was incubated for 6 h at 37°C in 5% CO2.

Then, 50 μL of inoculated bacteria were cultured on chocolate agar that contained 300 μL bacitracin and incubated overnight at 37°C in a CO2 incubator.

To increase the chances of S. pneumoniae isolation, 200 μL of STGG transporter medium was added to Todd–Hewitt broth (Merck, Darmstadt, Germany) that contains 1% of both glucose and yeast extract. Then, it was incubated for 6 h at 37°C in 5% CO2. Then, 50 μL of this medium was cultured on tryptic soy agar (CONDA, Madrid, Spain) (containing 5% sheep blood, and 5 mg/L gentamycin) overnight at 37°C with 5% CO2. Identification of S. pneumoniae and H. influenzae was then performed according to the manual for laboratory identification [10].

DNA extraction

DNA was extracted by DNA extraction kit according to the manufacturer’s instructions (BioFact, Daejeon, South Korea). DNA purity, quality and quantity were measured by absorbance spectrophotometry (Nanodrop-1000; NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. Finally, extracted DNAs were stored at –20°C for further processing.

Primer designing

The oligo analysis and design program (OLIGOWARE 3.0) were used for designing the 16S rRNA (species-specific internal fragment), virulence factor (ply and hpd genes) and iron uptake (tbpA and piuA genes) primer sequences (Table 2). The basic local alignment search tool (BLAST) was used to evaluate the specificity of the primer sequences. The primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

Table 1. The summary of demographic characteristics of healthy children in 37 Streptococcus pneumoniae and 21 Haemophilus influenzae isolates

| Characteristics                       | Streptococcus pneumoniae n (%) | Haemophilus influenzae n (%) |
|---------------------------------------|-------------------------------|-----------------------------|
| Sex                                    |                               |                             |
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Table 2. The primer sequences designed and used in the present study

| Target gene | Primer sequences (5’ to 3’) | Product length (bp) | Ref. |
|-------------|-----------------------------|---------------------|------|
| 16SrRNA S. pneumoniae | F- GCATAATTTGTTTCCCAGATG | 100 | present study |
|             | R- ATCAAGCAGCTGCGCTCATCA    | 77  | present study |
| piuA        | F- GCACCTCTGGAATTACGCC    | 112 | present study |
|             | R- CTAGGGCCACATCCCTGCC     | 140 | present study |
| tpbA        | F- ACCAAGAGTGGTGGTCCTGCCT | 136 | present study |
|             | R- GGATGGCTCTGAGTGTTGGC     | 135 | present study |
| hpd         | F- GTATGGCTTGGATGTTGGCA     | 121 | study       |
Direct detection of S. pneumoniae and H. influenzae by RT-qPCR
For direct identification of S. pneumoniae and H. influenzae, RT-qPCR was applied. For this purpose, the specific region of 16S rRNA was used. Molecular detection of 16S rRNA was carried out by real-time PCR. Each reaction was run in triplicates, directly from swab samples. The reaction mixture was prepared in a total volume of 25 μL, containing 1 μL of each forward and reverse primer, 12.5 μL of 2 × Real-Time PCR Master Mix (SYBR Green; Solis BioDyne, Tartu, Estonia), 7.5 μL of nuclease-free water, and 3 μL of template DNA.

A real-time PCR assay for S. pneumoniae was performed by the following protocol: an initial holding at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 58.5°C for 30 seconds, extension at 68°C for 30 seconds. In addition, a real-time PCR assay for H. influenzae was performed by the following protocol: an initial holding at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 58.6°C for 30 seconds and extension at 68°C for 30 seconds. Streptococcus pneumoniae (ATCC 33400) and H. influenzae (ATCC 10211) strains were used as positive controls. The negative control included all the ingredients of the reaction mixture except the DNA template. The reaction was run on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

Molecular detection of iron uptake and virulence factor genes
PCR was performed to investigate the virulence factors (ply and hpd) and iron uptake (tbpA and piuA) genes among positive cultures of S. pneumoniae and H. influenzae. The reaction mixture contained 1 μL of each forward and reverse primer, 12 μL of Hot Start 2 × Master Mix (Ampliqon, Odense, Denmark), 8 μL of nuclease-free water and 3 μL of DNA template. A PCR assay was performed using the following protocol: an initial holding at 95°C for 5 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 1 min, with final extension at 72°C for 1 min. The reaction was run in a PCR thermal cycler C1000 (Bio-Rad). Then PCR products were analysed by Gel document, Bio-Rad’s Gel Doc XR + system. The 1.5% agarose gel was run under standard conditions and stained with Simply Blue Safe Stain (Invitrogen, Carlsbad, CA, USA).

Sequencing analysis
The amplified products of iron uptake and virulence factor genes were subjected to DNA sequencing by Bioneer (Daejeon, South Korea). The obtained sequences were analysed by Chromas 2.5 software (Technelysium, Tewantin, Australia; http://technelysium.com.au/wp/chromaspro/). Finally, the sequences were evaluated using the BLASTn program at the NCBI database (https://www.ncbi.nlm.nih.gov/pubmed/).

Statistical analysis
All data obtained from culture, sequencing and questionnaires were analysed by SPSS software (19.0). Chi-squared test was used to determine the correlation between variables. A p value < 0.05 was considered statistically significant.

Results
Detection of S. pneumoniae and H. influenzae based on culture procedures
Results of conventional methods revealed that 14/123 (11.4%) and 8/123 (6.5%) of the isolates were identified as the S. pneumoniae and H. influenzae, respectively. Also, these isolates were confirmed by the detection of 16S rRNA gene.

Significant detection of S. pneumoniae and H. influenzae by RT-qPCR
Among 123 samples, detection of S. pneumoniae and H. influenzae was performed by RT-qPCR via specific regions of 16S rRNA. The results indicated that 37 samples were positive for S. pneumoniae and 21 samples were positive for H. influenzae.

The prevalences of S. pneumoniae and H. influenzae were 35% and 10% in winter and 27.7% and 20.5% in spring, respectively. According to the statistical analysis results, seasonal sampling had no significant effect on the frequency of S. pneumoniae and H. influenzae (p 0.11).

Investigation of genes involved in iron uptake and pathogenicity
According to the important role of iron in survival and growth of pathogenic bacteria, tbpA and piuA, genes involved in iron acquisition, were monitored by PCR-based sequencing in H. influenzae and S. pneumoniae, respectively.

Surprisingly, we found that all isolates carried tbpA and piuA genes. Also, among 37 isolates that were identified as S. pneumoniae, 30 were positive for ply gene, which encodes pneumolysin. In addition, 19 isolates were positive for hpd, which encodes protein D. This was confirmed by sequencing analysis.

Discussion
There is continuous intense competition between pathogenic bacteria and the microbiota in the body [11]. Therefore, several studies showed that destruction of the microbiota can lead to infections by pathogenic bacteria such as H. influenzae and S. pneumoniae [12–14]. In fact, determination of the carrier rate...
of pathogenic bacteria that are potentially able to create respiratory infections is difficult. The carrier rates of the mentioned pathogens are highly dependent on socio-economic status, environmental conditions, age and antimicrobial therapy of the population.

As mentioned, S. pneumoniae and H. influenzae often colonize the human upper respiratory tract. These pathogens are simply transmitted from colonized/infected individuals to the healthy population through respiratory droplets or oropharyngeal secretions. Invasive infections may occur by potential pathogens through asymptomatic colonization.

Between 7% and 99% of S. pneumoniae and 5% and 87% of H. influenzae carriers are associated with age, physical health and economic status of children [15].

In the present study, there was no statistically significant difference between variables including, sex, history of hospitalization, history of chronic respiratory diseases, method of delivery (cesarean section or vaginal delivery), the number of family members, smoking by other family members, feeding of infants in the first 2 years of life (breastfeeding or formula feeding or both) with frequency of H. influenzae and S. pneumoniae (p ≥ 0.05). However, there was a statistically significant difference between sex and the frequency of H. influenzae and S. pneumoniae (p 0.05).

Fastidious bacteria are commonly hard to identify using culture-based methods because they are difficult to perform (must be performed by trained personnel), time-consuming and laborious. In addition, culture-based methods are often less sensitive than molecular assays.

Several methods are available for the characterization of pathogenic bacteria but molecular and culture procedures are more important than others. In the current study, we identified 37 S. pneumoniae and 21 H. influenzae isolates by real-time PCR, but only 14 S. pneumoniae and eight H. influenzae using culture methods. These findings demonstrated the superiority and importance of molecular methods in comparison with culture-based methods.

This technique can provide a reliable method for identifying pathogenic bacteria such as H. influenzae and S. pneumoniae [16,17].

In line with our study, Xu et al. [18] evaluated the nasopharyngeal and oropharyngeal samples of 320 children aged 6–24 months for the presence of H. influenzae and S. pneumoniae using culture and phenotypic detection methods. The prevalence of S. pneumoniae and H. influenzae were 30.3% and 11.7% in healthy children and 52.7% and 47.9% in children with acute otitis media, respectively, which is consistent with the findings of the present study.

On the other hand, the presence of abundant iron acquisition (100% of isolates) and virulence (81% of ply and 90% of hpd) genes among the isolates could be a warning for the creation of infectious and transmitted diseases among children.

Also, we found 12 cases of simultaneous colonization of H. influenzae and S. pneumoniae, which highlights the importance of this problem.

The presence of iron acquisition genes is essential for the pathogenesis of H. influenzae and the survival of S. pneumoniae in the body. All isolated bacteria were positive for the presence of these genes in the present study. Moreover, 81% of the S. pneumoniae isolates had the ply gene and 90.5% of the H. influenzae isolates had the hpd gene. The selected virulence genes of this study are mostly associated with respiratory diseases and dysfunctionality of respiratory cells. Therefore, it can be concluded that children who carried the mentioned bacterial pathogens that possess those genes are at greater risk of developing respiratory infections.

Conclusions

We suggest monitoring of children to identify H. influenzae and S. pneumoniae carriers. This would be helpful for the prevention of serious respiratory infections among children. Certainly, after identification, carriers should be treated.

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Ethical approval

This project was approved by the Ilam University Human Ethics committee.

Conflict of interest

There are no conflicts of interest.

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