ORIGINAL ARTICLE

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. **T**his 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.

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

There is a direct correlation between asymptomatic nasal or throat colonization by pathogenic bacteria with many respiratory diseases [1]. Human pathogenic bacteria use a variety of elements, such as iron, which plays a key role in survival and growth [2,3].

Therefore, pathogenic bacteria that encode genes involved in iron acquisition could be threatening for public health, especially in children [4]. The importance of nasopharyngeal/oropharyngeal carriers is due to their association with the progress of disease, as well as distribution and transmission of pathogens [5]. Accordingly, among pathogenic bacteria that are able to colonize the noses and throats of children, *Streptococcus pneumoniae* and *Haemophilus influenzae* cause serious diseases including meningitis, otitis media, bacteraemia and pneumonia [6]. The presence of *H. influenzae* and *S. pneumoniae* in the throats of children have outstanding importance in the spread of these pathogens in families, schools and daycare centres [7].

Streptococcus pneumoniae produces several virulence factors for survival or invasion of the human host cell. One of the major virulence factors of *S. pneumoniae* is a pneumolysin that has multiple functions, such as blocking of respiratory ciliary motion, inhibition of bactericidal action of polymorphonuclear leucocytes and synthesis of antibodies [8]. Also, the 42-kDa D protein exists in both type b and encapsulated *H. influenzae*, which is valuable for the identification of carriers and vaccine research [9]. The simultaneous colonization by *S. pneumoniae* and *H. influenzae* indicates the importance of monitoring healthy children to identify carriers.

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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		
Male	19 (31.7)	11 (18.3)
Female	18 (28.6)	10 (15.9)
Age groups (years)		
2–3	19 (51.35)	11 (52.4)
3–4	4 (10.8)	5 (23.8)
4–5	3 (8.1)	2 (9.5)
5–6	11 (29.8)	3 (14.3)
Delivery method		
Caesarean section	20 (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		
Winter	14 (37.8)	4 (19)
Spring	23 (62.2)	17 (81)

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 cavity 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.

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- GCATAATTTGTTTGCGGATG R- ATCAAGCACTCGCTCATCA	100	present study
16SrRNA H. influenzae	F- GCACTTCTGGAATTAACGC R- AGGGCTATTGCAGCAAACTT	77	present study
piuA	F- CAAGCAAGCAAGGACGACTA R- CTAGCTTGGCCAATTCTTCC	112	present study
ply	F- CCTCAGACAGAGTGGAAGCA R- AAGCGACTGCCTTCTTGAAT	140	present study
tbpA	F- ACCAAGAGTTTGGTCTCGCT R- CCATAATTGCCCTTTCCAGT	136	present study
hpd	F- GATGGCTTGACTGATGTTGC R- TTTGCCATCTTTGGTTTCAA	135	present study

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% CO_2.

Then, 50 µL of inoculated bacteria were cultured on chocolate agar that contained 300 µL bacitracin and incubated overnight at 37°C in a CO_2 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% CO_2. 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% CO_2. 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).

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 (caesarean 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* ($p \geq 0.05$). 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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