ORIGINAL ARTICLE

Asymptomatic carriers of *Neisseria meningitidis* and *Moraxella catarrhalis* in healthy children

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Abstract

There is a close correlation between asymptomatic oropharyngeal colonization by bacterial pathogens and paediatric respiratory diseases. Evaluation of the frequency of asymptomatic carriers of *Neisseria meningitidis* and *Moraxella catarrhalis* in healthy children was the main aim of the current study. In this cross-sectional study, 123 oropharyngeal swabs were collected from children between 2 and 6 years old in kindergartens of Ilam, Iran. *Moraxella catarrhalis* and *N. meningitidis* were identified using phenotypic and genotypic assays. In addition, the occurrence of the virulence factors (*ctrA* and *uspA1*) and iron uptake (*tbpA*) genes was evaluated by PCR. Results showed that 21 *M. catarrhalis* isolates and 17 *N. meningitidis* isolates were identified by conventional microbiological and biochemical methods, but the RT-PCR assay detected that 18 and 8 isolates were positive for *M. catarrhalis* and *N. meningitidis*, respectively. The *tbpA* gene was positive in all *N. meningitidis* and *M. catarrhalis* isolates. Seven isolates were positive for the *ctrA* gene in *N. meningitidis* and seven isolates were positive for the *uspA1* gene in *M. catarrhalis*. These pathogenic bacteria often occurred as asymptomatic carriage of *N. meningitidis* in children from large families with low economic status, which reflects the importance of the environment and socio-economic level of families in the distribution of these potentially pathogenic bacteria in the oropharynx of children. Monitoring for the carriage of potential pathogenic bacteria in the nasopharynx of healthy children is important as this can predispose to infectious diseases; common exposure to human respiratory bacterial pathogens is a further risk factor.

Keywords: Asymptomatic carriers, *ctrA*, *Moraxella catarrhalis*, *Neisseria meningitidis*, *tbpA*, *uspA1*, virulence factors
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Introduction

Within the first week of life the microbiome of newborns develops in different parts of the body, such as the respiratory tract. Any damage to or changes in this microbiome could be considered as risk factors for colonization by pathogenic bacteria, followed by respiratory disease. Birth is considered the initiating point of development of the microbiota and during the first week of life the microbiome develops throughout the body, including the respiratory tract [1]. Several factors are effective in the formation of the respiratory microbiome, among which genetic factors as internal agents and type of delivery as an external agent are more important than others factors [2]. For example, the dominant population of bacteria in the microbiome of children born by vaginal delivery is like the microbiome of the mother's vagina, and includes *Lactobacillus*, *Prevotella* and *Sneathia*. In contrast, in children delivered by caesarean section the dominant bacterial population of the microbiome comprises *Staphylococcus*, *Corynebacterium* and *Bifidobacterium*. The composition of the respiratory microbiome among breastfed children is different from that of children given powdered milk [3]. Colonization of the nasopharynx by potential pathogenic bacteria introduces bacterial invasion. However, disease develops in only a small group of individuals who are colonized [4–6]. Generally, the microbiome formed in

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the early stages of life determines the composition of the microbiome in later stages of life [1]. The importance of the microbiome to public health is linked to its colonization by bacterial pathogens [7]. Therefore, damage to or changes in this microbiome could be considered as risk factors for the creation of some dangerous respiratory diseases [8]. Accordingly, colonization by some bacterial pathogens such as *Moraxella*, *Neisseria*, *Haemophilus* and *Corynebacterium* could lead to the creation of dangerous infections in the respiratory tract of children and adults [9]. Successful mucosal colonization by and survival of respiratory pathogens through person-to-person transmission is essential and a necessary stage in the development of clinical diseases. Over the past two to three decades, *Moraxella catarrhalis* has become a true pathogen [10]. Infants, in the early stage of life are soon exposed to *Moraxella*, but the rate of carriers in older children and healthy adults is low [11]. One of the main sources of *M. catarrhalis* is transmission to newborns from adults, which explains the premature exposure of infants and reflects the common colonization through very close contact with adults [12]. Meningococcal disease is a major cause of child morbidity and mortality around the world, although diagnosis and treatments are improving. The usual sources of meningococcal infection are asymptomatic human nasopharyngeal carriers [13]. The overall nasopharyngeal carriage rate for *Neisseria meningitidis* varies from <2% to 15% in healthy children in different geographic regions [4,14–17]. The aim of this study was to characterize the asymptomatic carriers of *N. meningitidis* and *M. catarrhalis* in healthy children of Ilam aged between 2 and 6 years using phenotypic and genotypic assays.

Methods

Oropharyngeal sampling, processing and isolation

This cross-sectional study was conducted from 2015 until 2016 on 123 oropharyngeal swabs that were collected from the oropharynx among children between the ages of 2 and 6 years in kindergarten of Ilam, Iran. After obtaining verbal and written consent from parents and filling out the questionnaire with the child's parents, the sampling was performed. Inclusion criteria in this study were that children should be healthy and not have received antibiotics in the last month. This study has been approved by the ethics committees of the Ilam University of Medical Sciences. The oropharynx samples from posterior the pharynx and the back of the tongue were obtained by trained staff using sterile Dacron-tipped swabs (BD Biosciences, Frankland Lakes, NJ, USA) immediately transported to skimmed milk–tryptone–glucose–glycerol medium (Merck, Darmstadt, Germany), and plated directly onto Thayer–Martin agar (HiMedia, Mumbai, India) plates, and incubated at 37°C in 5% CO_2 for 72 hours. Identification of *M. catarrhalis* and *N. meningitidis* was performed according to the manual for laboratory identification [18–20].

Molecular characterization

DNA extraction. The DNA was extracted using a Bacterial DNA isolation kit according to the instructions of the manufacturer (Denazist Asia, Mashhad, Iran). DNA purity, quality and quantity were measured by absorbance spectrophotometry (Nanodrop-1000; NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis. The DNA was stored at −20°C for further analysis.

Molecular detection of M. catarrhalis and N. meningitidis. For molecular detection of *M. catarrhalis* and *N. meningitidis* a real-time PCR assay was carried out in triplicate. The conserved domain of the 16s rRNA gene was targeted. The primer sequences are listed in Table 1. A Real-Time PCR SYBR Green Kit (Solis BioDyne, Tartu, Estonia) was used with the following cycle conditions: an initial holding at 95°C for 3 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds, and annealing at 58.5°C (*M. catarrhalis*) or 59.5°C (*N. meningitidis*) for 30 seconds, and extension at 69°C for 30 seconds. The reaction mixture, in a total volume of 25 μL, included 1 μL of each forward and reverse primer (5 pmol/mL), 12.5 μL of 2 × Real-Time PCR Master Mix (SYBR Green; Solis BioDyne), 7.5 μL nuclease-free water and 3 μL of template DNA. In this study, *N. meningitidis* ATCC 13090 and *M. catarrhalis* ATCC 25240 were used as the positive controls. Also, negative controls (all the elements of the reaction mixture without template DNA) were performed. The reaction was run on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

Virulence genes amplification. To evaluate the virulence factors involved in iron acquisition (*tbpA* gene) in both bacteria, and the meningococcal capsule production (*ctrA* gene) and adhesion factor in *M. catarrhalis* (*uspA1* gene) the PCR assay was performed on the isolates that were positive for *M. catarrhalis* and *N. meningitidis* by real-time PCR. PCR assay was carried out with the following cycle conditions: an initial holding at 95°C for 5 min, followed by 35 cycles of 94°C for 45 seconds, 58.5°C for 45 seconds and 72°C for 1 minute, with final extension at 72°C for 10 minutes. The reaction mixture included 1 μL of each forward and reverse primer, 12 μL of Hot Start 2 × Master Mix (Avagene, Tehran, Iran), 8 μL nuclease-free water and 3 μL of template DNA. The reaction was run on a PCR thermal cycler C1000 (Bio-Rad) and PCR products were analysed by Gel document (Bio-Rad's Gel Doc XR + system). The primer sequences of are listed in Table 1.

TABLE 1. The sequences of primers used in this study

Genes	Primer sequences (5′–3′)	Product size (bp)	Ref.
16S rRNA *N. meningitidis*	F-ACGAATATGCGGTTACGTTG R-CCGTCTTTCGGGTTAAGAAG	127	This study
16S rRNA *M. catarrhalis*	F-AGCGATACCAAACTGTGCTG R-ATGATGAGCTGGGTCGTACA	130	
uspA1 M. catarrhalis	F-TTTGATGATGTGCGTGTGTC R-TGTCAATGTGCGGATAGGTT	94	
tbpA M. catarrhalis	F-CAGTGGGTCGTTTGAAGCTA R-CCGACATTAACGATTTGACG	115	
ctrA N. meningitidis	F-GGCAGAGATTCCAACGGTAT R-GAATTTCTGCACTTCAGCCA	133	
tbpA N. meningitidis	F-ACGCAACAAACTTTCGACAC R-GGTAAACAGTCCGCCGTATT	144	

Statistical analysis. To determine the correlation between the variables, the χ^2 test (as a univariate test) was used by SPSS software 19.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

Results

Phenotypic identification

The results for phenotypic identification of *N. meningitidis* and *M. catarrhalis* among 123 Dacron swab samples showed that 17 and 21 isolates were identified as *N. meningitidis* and *M. catarrhalis*, respectively.

Molecular detection of N. meningitidis and M. catarrhalis

Real-time PCR assay indicated that 8 and 18 isolates were positive for *N. meningitidis* and *M. catarrhalis*, respectively. Results showed that the *tbpA* gene was positive in all *N. meningitidis* and *M. catarrhalis* isolates. Also, seven isolates were positive for the *ctrA* gene in *N. meningitidis* and seven isolates were positive for the *uspA1* gene in *M. catarrhalis*. The demographic characteristics of the healthy children with eight *N. meningitidis* and 18 *M. catarrhalis* isolates are displayed in Table 2.

Correlation of variables with the frequency of N. meningitidis and M. catarrhalis in the oropharynx of children

There was no significant relationship between the frequency of these bacteria with gender (*N. meningitidis* p 0.264 and *M. catarrhalis* p 0.564), child's age (*N. meningitidis* p 0.357 and *M. catarrhalis* p 0.628), sampling period (*N. meningitidis* p 0.567 and *M. catarrhalis* p 0.515), hospitalization history of the child (*N. meningitidis* p 0.535 and *M. catarrhalis* p 0.558), birth method (*N. meningitidis* p 0.595 and *M. catarrhalis* p 0.282), and child nutrition (*N. meningitidis* p 0.601 and *M. catarrhalis* p 0.288).

TABLE 2. Summaries of demographic characteristics of children in eight *Neisseria meningitidis* and 18 *Moraxella catarrhalis* isolates

Characteristics	*N. meningitidis n* (%)	*M. catarrhalis n* (%)
Sex		
Male	3 (31.7)	5 (28.6)
Female	10 (18.3)	8 (15.9)
Age groups (years)		
2–3	3 (51.35)	3 (52.4)
3–4	1 (10.8)	2 (23.8)
4–5	3 (8.1)	3 (9.5)
5–6	1 (29.8)	10 (14.3)
Method of birth		
Caesarean section	5 (54)	3 (46)
Vaginal	10 (62)	8 (38)
Feeding in the first 2 years of life		
Breastfeeding	6 (81)	17 (90.5)
Milk powder	0 (2.7)	0 (0)
Both of them	2 (16.3)	1 (9.5)
History of hospitalization	2 (27)	4 (23.8)
Sampling		
Winter	3 (37.8)	6 (19)
Spring	5 (62.2)	12 (81)

There was a significant correlation between the income of the children's family and the frequency of *N. meningitidis* in the oropharynx; frequency of *N. meningitidis* was higher in children living in poorer economic conditions (p 0.041). In this study there was no significant relationship between the income of the family of children and the frequency of *M. catarrhalis* in the oropharynx (p 0.242). Statistical analysis showed that there was a significant relationship between the number of family members of the child and the frequency of *N. meningitidis*, but there was no significant relationship between the number of family members and the frequency of *M. catarrhalis* (p 0.293).

Concurrent colonization of N. meningitidis and M. catarrhalis in children's oropharynx

Three children were positive for the presence both of *N. meningitidis* and *M. catarrhalis*.

Discussion

Microbial communities have inhabited the human body for millions of years [21]. One of the most important mucosa in humans is that of the respiratory tract, which plays a major role in protecting human health [22]. One of the most important roles of the respiratory microbiota in human health is to prevent colonization by pathogenic bacteria. Asymptomatic carriage of potential pathogens may lead to allergic disorders and invasive infections [23–25]. Many factors affect nasopharyngeal colonization rates in different geographical regions including methodological procedures, genetic background variables and socio-economic status, such as age, season, acute respiratory illness, diet (breast-feeding versus bottle-feeding), sleeping position, housing, access to health care, poor

hygiene, the number of family members, overcrowded living circumstances, day-care contact and number of siblings [4]. These differences imply that factors that impact colonization are various and not certain. In a recent published study [26], conducted in healthy children to characterize the bacterial communities of their respiratory microbiome using oropharyngeal swabs, traditional culture and Sanger sequencing of the 16S rRNA gene, 346 bacterial isolates belonging to three phyla and nine families were categorized. In another report conducted in healthy school students [27], among 1813 oropharyngeal swabs investigated by traditional culture and culture-amplified PCR, 320 were positive by at least one of the three methods of which only one was positive only by culture, the other 133 culture-positives were also positive by culture-amplified PCR. Hence, although a significant use of obtaining, speciating and saving pure isolates when performing cultures from carriage reports relates to the further categorization that can then be performed, including whole-genome sequencing, such high-cost and time-consuming processing can be reserved for the minority of samples that test positive by one or other of the PCR methods as long as swab samples have been collected into glycerol-containing storage media, such as skimmed milk–tryptone–glucose–glycerol medium, so that pure isolates can be prepared when required [27]. In a recent study [28], Rodrigues et al. collected oropharyngeal swabs and saliva samples from 1005 healthy students in Portugal. The outcomes of this research demonstrated that viable meningococci can be detect in the saliva of carriers using direct and culture-amplified PCR. Culture-amplified PCR improves the sensitivity of detection in samples when combined with direct PCR detection. Use of these methodologies should significantly increase the power of carriage reports to detect the impact of vaccines upon carriage and transmission. The high importance of investigating the rates of asymptomatic carriage of potentially pathogenic agents, such as *Neisseria* and *Moraxella*, in the oropharynx of children is linked to the complex interactions that they have with the symbiotic bacteria. *Moxarella catarrhalis* is often carried symbiotically in the nasopharynx of individuals, especially asymptomatic children, but these pathogens have the capacity to cause multiple diseases of the upper respiratory tract, including sinusitis, laryngitis and acute middle ear infections in children and to intensify chronic obstructive pulmonary disease in adults [29]. The rate of nasopharyngeal carriage of *M. catarrhalis* is very high in children (>75%). In our study, the prevalence of *M. catarrhalis* was 14.6%, which is lower than expected compared with the estimated number of carriers of *M. catarrhalis* in children in another study [30]. However, as shown by Verhaegh et al., 6 months is the age at which a child has the highest susceptibility to colonization by *M. catarrhalis* of the respiratory tract; this sensitivity decreases with increasing age [31]. The only ecological status of *N. meningitidis* that threatens human health is the nasopharyngeal mucosa [32]. Colonization of the respiratory tract is a common strategy for asymptomatic carriage of a pathogenic agent. In this study, the frequency of *N. meningitidis* was 6.5%. Similar studies have been conducted in Asia. In a study by Kadayifci et al. in Istanbul, the prevalence of oropharyngeal carriage of *N. meningitidis* in 1000 healthy people aged 9–79 years was studied. *Neisseria meningitidis* was isolated from 6 out of 1000 people and meningococcal carrier age was between 21 and 40 years [33].

Asymptomatic carriers of *N. meningitidis* in the oropharynx of individuals are a risk for the spreading of pathogenic strains, especially in hospitals [34]. The significance of this concern will be raised when children who carry these pathogens are asymptomatic, that can be the propagator source of disease in the community. Thus, the timely identification and treatment of these pathogenic carriers can contribute to the health of the community.

In line with another study [35], we found correlation between higher carriage of *N. meningitidis* and higher number of family members. An interesting point in this study was the simultaneous colonization by *Neisseria* and *Moraxella* in the oropharynx of three children. Interestingly, these bacteria were also positive in terms of the presence of iron and virulence genes. The relative economic weakness of the families of these three children was notable; this variable can affect the colonization of most pathogenic agents in the oropharynx of children. Because a weaker economic situation causes poor health in children, it maybe lead to the formation of unsuitable microbiota. Identification of the encoding gene of the *N. meningitidis* capsule (*ctrA*) is an important indicator of the pathogenicity of *N. meningitidis* in terms of transmission by asymptomatic carriers to people at risk and the creation of meningococcal disease, In the present study, most of the detected *Neisseria* had this gene (87.5%) and there was significant correlation with the frequency of *N. meningitidis* in the oropharynx of these children. Perhaps one of the reasons for not recognizing this gene in 12.5% of the remaining positive samples was the presence of capsule serotypes other than type B. UspA1 as the main adhesion protein of *M. catarrhalis* has the main role in bacterial colonization of the mucosal epithelium and the sustainability of this pathogen is due to its binding to the extracellular matrix in the oropharynx of carriers. In the present study, the *uspA1* gene was evaluated and 38.9% of the identified *Moraxella* were positive for this gene. Perhaps the reason why this gene was not present as the main adhesion in 61.1% of the remaining isolates is the presence of *uspA2* and *uspA2H* genes in the isolates.

Conclusion

It is difficult to determine carriers of potential respiratory pathogens, because carriage is influenced by several factors

such as geographical area, sampling site, sampling technique and immunity schedule, socio-economic conditions. According to the results of this study, asymptomatic colonization of *M. catarrhalis* in the children's oropharynx is more common than of *N. meningitidis*. In general, there are no significant differences in the distribution of *Moraxella* and *Neisseria* in different geographical areas, and the relationship between age and season has been noted. In the present study, there was only a meaningful and significant relationship between the frequency of *Neisseria* in the oropharynx of a child and the poor economic status of the child's family and the presence of the child in a large family. Finally, it is suggested that because of the widespread and controversial nature of the issue of respiratory microbiota, more studies are needed using both nasopharyngeal and oropharyngeal swabs, asymptomatic placement and determination of microbiota profiles using sequencing in children.

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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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