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Distribution of Virulence Genes in *Moraxella catarrhalis* Isolated from Clinical Samples in the North of Iran

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Abstract

Background: *Moraxella catarrhalis* is an opportunistic bacterium that has pathogenicity in the human respiratory tract. The rates of colonization and infection of this bacterium are high in the respiratory tract of children and the elderly.
Methods: In this descriptive cross-sectional study conducted from April 2016 to March 2018, 400 samples were collected from patients with pharyngitis (n = 92), sinusitis (n = 85), otitis media (n = 43), respiratory failure (n = 60), and healthy individuals as the control group (n = 120) in the Northern part of Iran. The isolates were identified by phenotyping methods. Finally, the virulence genes in the isolates were detected by polymerase chain reaction (PCR) technique.
Results: Out of a total number of 400 samples, 32 samples (8%) were positive for *M. catarrhalis*. The frequency of this bacterium was found to be different in patients with pharyngitis as 5.44%, sinusitis as 8.24%, otitis media as 13.95%, respiratory failure as 15%, and in the control group as 4.16%. Since the P value from the Chi-square test was higher than 0.05, there was no relationship between the prevalence of *M. catarrhalis* and the type of the disease and control group. The PCR results showed that 100% of the isolates had *mcaP*, *ompJ*, and *ompCD* genes. However, the distribution of other virulence genes was varied.
Conclusions: Overall, our findings verified the existence of *M. catarrhalis* in patients with respiratory diseases. Therefore, the rapid identification and successful treatment can play an important role in preventing their spread. In addition, the results verified the high prevalence of virulence genes in *M. catarrhalis* isolates from patients compared to controls. Regarding the results of this study and by comparing with similar studies, it can be concluded that the frequency of pathogenic isolates may be different even in regions that are not geographically different.

Keywords: Colonization, Gene, Respiratory Tract Infection, *Moraxella (Branhamella) catarrhalis*, Polymerase Chain Reaction, Virulence

1. Background

In recent years, *M. catarrhalis* has emerged as a new pathogen and it is now considered an important cause of upper and lower respiratory tract infections in children and the elderly. *M. catarrhalis* is the third most commonly associated cause of otitis media in the world after *Streptococcus pneumonia* and *Haemophilus influenzae*. It is also the second most common cause of chronic respiratory tract obstruction (COPD), along with *S. pneumonia*. In addition, the bacterium is the cause of sinusitis, meningitis, conjunctivitis, endocarditis, bacteremia, septic arthritis, osteomyelitis, wound infection, and hospital infection. *M. catarrhalis* is the most common bacterium isolated from sputum, middle ear, sinus, oral, and throat swabs (1-3).

Since *M. catarrhalis* has been considered a harmless commensal bacterium for a long time, there is relatively little cognition about pathogenicity characteristics and its virulence factors. Generally, the pathogenicity of this bacterium, like other microorganisms, depends on the ability to escape from the host defense mechanisms and binding to cellular and mucus layer, as well (4). *M. catarrhalis* can attach to various types of cells, including bronchial epithelial cells, epithelial cells of the small airway, and type 2 alveolar cells (5, 6).

The adhesion factors of this bacterium that play a role in biofilm formation include Outer Membrane Proteins (OMPs), ubiquitous surface protein A (UspA), Hemagglutinin/*Moraxella catarrhalis* Immunoglobulin D-Binding Protein (MID/Hag), and *M. catarrhalis* Adherence Protein (McaP). Moreover, with the help of *M. catarrhalis* outer

membrane protein B (copB), it is able to extract iron from the host cells (1, 7).

Despite the important role of this bacterium in human health, many laboratories do not usually report *M. catarrhalis* as a true pathogen when compared to S. pneumonia and H. influenza; so, epidemiological studies have been conducted on *M. catarrhalis*. In addition, the *M. catarrhalis* colonization may vary based on the geographical area, living conditions, smoking status, health conditions, and other factors (8).

2. Objectives

The aim of this study was to investigate the frequency of *M. catarrhalis* in patients with respiratory infections relative to a control group and determine the frequency of responsible virulence genes in isolated strains.

3. Methods

3.1. Sample Collection

In this descriptive cross-sectional study conducted from April 2016 to March 2018, sampling was performed on 92 patients with sore throat, 85 patients with chronic sinusitis, and 43 patients with otitis media from throat swabs, sinus secretion, and ear discharge, respectively. The patients were selected from among those referring to the infectious wards of government hospitals in cities of Western Mazandaran province in the North of Iran (Noshahr, Tonekabon, and Ramsar) after being diagnosed by specialists.

In addition, lung secretions of 60 patients with respiratory failure in the intensive care unit of the hospitals connected to ventilators for 48 hours were collected. Further, the sampling of nasal secretions and throat swabs was performed on 120 healthy people without symptoms of sore throat, sinusitis, and ear infection. The sample size was calculated using this formula:

$$n = \frac{z^2 pd}{d^2} \quad (1)$$

The inclusion criteria of the study included patients diagnosed with respiratory infections and one of the symptoms of a sore throat, middle ear infection, sinusitis, and respiratory failure by infectious disease specialists. Patients who were hospitalized the week before the sampling, those who had received antibiotics one week before the sampling, patients with underlying illness, chemotherapy, and immune deficiency were excluded.

The study was approved by the National Ethics Committee for Biomedical Research (Code of Ethics: IR.IAU.TON.REC.1397.022), and written consent was obtained from all participants. For the epidemiological study, the data for each person including age, gender, and history of antibiotic consumption in the last month were recorded.

Following the sample collection, the specimens were immediately inoculated in brain heart infusion broth, transferred to a private medical diagnostic laboratory (pasture-Tonekabon), and placed in a 37°C incubator for 24 hours for initial enrichment.

3.2. Isolation and Identification of M. catarrhalis

The isolation method of *M. catarrhalis* was based on the standard culture technique. After the incubation time completed, each sample was cultured on blood agar (Merck, Germany). The culture medium was then placed in a 37°C incubator for 24 - 48 hours. After this period, the plates were evaluated for the phenotypic examination of *M. catarrhalis* including microscopic appearance, gram staining, catalase, oxidase, nitrate reduction, DNase tests, and fermentation of glucose, lactose, and sucrose.

3.3. Identification of Virulence Genes

All strains of *M. catarrhalis* were isolated and identified by the phenotypic method based on the polymerase chain reaction (PCR) technique. For this purpose, the DNA was extracted from the DNA extraction kit (Isogen, Russia). To determine the concentration and purity of DNA, the optical absorption of the sample was evaluated at 260 and 280 nm wavelengths. In order to implement the PCR and the amplification of the *M. catarrhalis* virulence gene, forward and rivers primers (Table 1) were synthesized by Tag Copenhagen (Denmark) as described by Verhaegh et al. (9) and Hays et al. (10).

To perform the molecular study, each reaction was performed in a total volume of 25 μL containing 13 μL of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 μL of 10 $\times$ PCR buffer (Geneall, Korea), 1 μL of 10 pmol of each primer, 1 μL of 10 mM dNTPs (Geneall, Korea), 0.5 μL of smart taq DNA polymerase (Geneall, Korea), 1 μL of 50 mM $MgCl_2$ (Geneall, Korea), and 5 μL of DNA template. The negative control tube contained the same PCR reagents as above-mentioned but had 5 μL of water substituted for the DNA template.

In the next step, the micro tip was mixed with the reaction mixture inside a thermal gradient cycler (Biorad, Germany). Then, PCR cycles started at 95°C for 45 s. The annealing temperature was set at 72°C for 60 s and then decreased by 1°C for each cycle until the temperature reached 55°C. The annealing temperature of 55°C was adjusted at 20 cycles, followed by the extension temperature of 72°C for 40 s and a final extension temperature of 72°C for 5 min.

Table 1. Forward and Reverse Primer Sequences for the Amplification of the *M. catarrhalis* Virulence Genes

Primer's Name	3' → 5' Sequence	Gene
mcaP-F	CGCAATAAAGATCACCATGCTTG	*mcaP*
mcaP-R	CGGGATCCCGCTGACACATTGCATTGATAAA	
MCA-F	CTAACGCTGCCATCAGCTAT	*ompJ*
MCAT2-R	GTTGCATTACGGCTGGTAAC	
Mcat.ompCD.F	ACGCACTGGCAAGAAGCTAGA	*ompCD*
Mcat.ompCD.R	GACCTGCACCAACCAAGACAT	
McatHag-F	GTCAGCATGTATCATTTTTTAAGG	*Hag*
McatHag-R	TGAGCGGTAAATGGTTTAAGTG	
McCOPB2-F	GGCGTGCGTGTTGACCGTTTTG	*copB*
McCOPB2-R	GTTTGGCAGGCGATAGGCGACAT	
MeierRT-F	CGTTATGCACTAAAAGAGCAGGTC	*uspA1*
MeierRT-R	GCATCTGACCAGCTTAGACCAATC	
uspA2-F	CGCTGTAACCAGTGCCATGA	*uspA2*
uspA2-R	ACGATAGCCAGCACCGATAG	

An aliquot of all PCR products was run on a 2% (w/v) agarose gel with a 100 bp DNA ladder (Fermentas, Germany) and electrophoresed at 75 V for 40 min. The bands were visualized using ethidium bromide staining and photographed by a UV-transilluminator (UV DOC, England). In order to confirm the molecular results, the PCR products of each gene were sent to the Macrogene Corporation (South Korea) for DNA sequencing. All sequences data were subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to definitively identify each gene amplicon.

3.4. Statistical Analysis of Data

In this study, Fisher and Chi-square tests in IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, N.Y., USA) were used to investigate the relationship between demographic factors and frequency of *M. catarrhalis* and the relationship between virulence genes and the type of diseases. The significance level of less than 0.05 was considered significant.

4. Results

4.1. Isolation of M. catarrhalis from Different Specimens

In this study, a total number of 400 samples were collected to identify *M. catarrhalis* in patients with respiratory diseases and healthy people during the period from April 2016 to March 2018. Overall, *M. catarrhalis* was present in 32 samples giving the frequency of 8%.

The frequency of this bacterium was found to be different in patients with pharyngitis as 5.44% (5 out of 92), sinusitis as 8.24% (7 out of 85), otitis media as 13.95% (6 out of 43), respiratory failure as 15% (9 out of 60), and in healthy controls as 4.16% (5 out of 120). The frequency distribution was analyzed by gender, age, and history of antibiotic use and the results can be seen in Table 2. The obtained data showed that 3.5% of women and 4.5% of men in the surveyed community were positive for *M. catarrhalis*. In addition, 1.25% of the patients under the age of 20, 1.75% in the age group of 21 - 40 years, 2% in the age group of 41 - 60 years, and 3% of the patients over the age of 60 were positive for this bacterium. In addition, 4.5% of the patients with *M. catarrhalis* had the history of antibiotic use in the month prior to sampling.

Since the P values from the Chi-square test for gender, age, antibiotic use history, and type of population under study were higher than 0.05, there was no relationship between the prevalence of *M. catarrhalis* and the type of disease and other demographic factors.

4.2. Evaluation of the Presence of Virulence Genes

The data obtained from the PCR and sequencing performed to determine the frequency of virulence genes in the isolated strains showed that 100% of the isolates (32 out of 32) had the *mcaP*, *ompJ*, and *ompCD* genes, 84.4% (27 of 32) had the *Hag* gene, 90.7% (29 of 32) had the *copB* gene, 96.9% (31 of 32) had the *uspA1* gene, and 78.2% (25 of 32) had the *uspA2* gene.

The frequency of different genes in the *M. catarrhalis* isolates in patients with respiratory infections and the con-

Table 2. The Frequency of *M. catarrhalis* Isolated from Samples of Patients with Respiratory Diseases and Healthy Controls[a]

Demographic Variable	Pharyngitis		Sinusitis		Otitis Media		Respiratory Failure		Control Group	
	Frequency	Positivity	Frequency	Positivity	Frequency	Positivity	Frequency	Positivity	Frequency	Positivity
Gender										
Female	43 (46.74)	2 (40)	46 (54.12)	3 (42.86)	28 (65.12)	4 (66.67)	33 (55)	4 (44.44)	56 (46.66)	1 (20)
Male	49 (53.26)	3 (60)	39 (45.88)	4 (57.14)	15 (34.88)	2 (33.33)	27 (45)	5 (55.56)	64 (53.34)	4 (80)
P value[b]	0.562		0.407		0.656		0.334		0.370	
Age, y										
< 20	11 (11.96)	2 (40)	8 (9.41)	2 (28.57)	2 (4.65)	-	5 (8.33)	-	14 (11.66)	1 (20)
21 - 40	25 (27.17)	1 (20)	37 (43.53)	3 (42.86)	5 (11.63)	1 (16.67)	9 (15)	1 (11.11)	44 (36.66)	1 (20)
41 - 60	30 (32.61)	1 (20)	29 (34.12)	1 (14.29)	21 (48.84)	4 (66.67)	12 (20)	2 (22.22)	37 (30.84)	-
> 60	26 (28.26)	1 (20)	11 (12.94)	1 (14.29)	15 (34.88)	1 (16.67)	34 (56.67)	6 (66.67)	25 (20.84)	3 (60)
P value[b]	0.320		0.226		0.600		0.942		0.066	
History of antibiotic use										
Yes	48 (52.17)	1 (20)	61 (71.76)	5 (71.43)	36 (83.72)	5 (83.33)	42 (70)	7 (77.78)	18 (15)	-
No	44 (47.83)	4 (80)	24 (28.24)	2 (28.57)	7 (16.28)	1 (16.67)	18 (30)	2 (22.22)	102 (85)	5 (100)
P value[b]	0.189		0.642		0.681		0.453		0.437	
Total	92 (100)	5 (5.43)	85 (100)	7 (8.23)	43 (100)	6 (13.95)	60 (100)	9 (15)	120 (100)	5 (4.16)

[a] Values are expressed as No. (%).
[b] $P < 0.05$ was considered significant.

trol group can be seen in Table 3. As seen, in all strains isolated from patients and the control group, the *mcaP*, *ompJ*, and *ompCD* genes were present with a frequency of 100%. This is while the abundance of other genes was varied. The obtained data showed that although the frequency of the *uspA1* gene was 100% in the patient group, the frequency of this gene (80%) was lower in healthy individuals (control group). In addition, the prevalence of *uspA2*, *Hag*, and *copB* genes in the healthy group (60%) was lower than the prevalence of the genes in *M. catarrhalis* isolated from patients with respiratory infections. Since the P value for each of the above genes from the Chi-square test is higher than 0.05, we can conclude that the relationship between the frequency of the virulence genes of *M. catarrhalis* and the type of the disease is not confirmed (Table 3).

5. Discussion

Given the fact that most respiratory infections are treated experimentally and without specific identification of the causative agent, the awareness of the prevalence of infectious organisms in each geographic region is important for an effective therapeutic approach. Since there is not enough information regarding the frequency and presence of pathogenic genes in *M. catarrhalis* in Iran, the present study can be considered a new study in the country.

As mentioned earlier, *M. catarrhalis* has the ability to colonize the respiratory tract without symptoms. Different studies have shown a high degree of *M. catarrhalis* colonization in children and healthy carriers. For example, Berner et al. (11), Masuda et al. (12), and Verhaegh et al. (9) reported the colonization rate of this bacterium in healthy children as 9%, 35%, and 29%, respectively. In the present study, the frequency of this bacterium in healthy carriers was estimated at 4.16%; the difference with previous studies may be due to the differences in geographic regions, seasons, and years of sampling.

In the present study, the prevalence of *M. catarrhalis* in patients with pharyngitis was 5.44%. Abuhammour et al. (13) also identified *M. catarrhalis* as one of the triggers for pharyngitis. Another study conducted by Heiniger et al. (14) for the identification of *M. catarrhalis* reservoirs in children's pharynx lymph tissue showed a frequency of 80%.

On the other hand, in this study, from among 85 patients with sinusitis, this bacterium was isolated and identified in seven patients giving a frequency of 8.24%. In sinusitis, both in acute and chronic conditions, *M. catarrhalis* is the third most common cause of infection, and its prevalence may be up to 20% (15). In a study conducted by Gergova et al. (16) to determine the prevalence of *M. catarrhalis* in patients with sinusitis, the prevalence of the bacterium was reported as 39%.

As mentioned above, *M. catarrhalis* is known to be the third etiologic agent in the development of middle ear infection (17). In a study conducted by Sillanpaa et al. (8), the prevalence of the bacterium was 18.91%, which indicated the important role of the bacterium in the disease. In this study, from among 43 patients with otitis media, the bacterium was isolated and identified in six patients, with a frequency of 13.95%.

M. catarrhalis is also described as one of the main

Table 3. The Comparison of the Frequency of Virulence Genes among the Population in Positive Samples[a]

Location of identification	*mcaP*	*ompJ*	*ompCD*	*Hag*	*copB*	*uspA1*	*uspA2*
Pharyngitis							
Positive	5 (100)	5 (100)	5 (100)	4 (80)	4 (80)	5 (100)	3 (60)
Negative	0 (-)	0 (-)	0 (-)	1 (20)	1 (20)	0 (-)	2 (40)
Sinusitis							
Positive	7 (100)	7 (100)	7 (100)	6 (85.71)	7 (100)	7 (100)	5 (71.43)
Negative	0 (-)	0 (-)	0 (-)	1 (14.29)	0 (-)	0 (-)	2 (28.57)
Otitis media							
Positive	6 (100)	6 (100)	6 (100)	5 (88.33)	6 (100)	6 (100)	6 (100)
Negative	0 (-)	0 (-)	0 (-)	1 (16.67)	0 (-)	0 (-)	0 (-)
Respiratory failure							
Positive	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	8 (88.89)
Negative	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	1 (11.11)
Control group							
Positive	5 (100)	5 (100)	5 (100)	3 (60)	3 (60)	4 (80)	3 (60)
Negative	0 (-)	0 (-)	0 (-)	2 (40)	2 (40)	1 (20)	2 (40)
P value	-	-	-	0.317	0.157	0.308	0.364

[a]Values are expressed as No. (%).

causes of lower respiratory tract infections. In this study, from among 60 patients with lower respiratory tract infection who were admitted to the intensive care unit, *M. catarrhalis* was isolated and identified in nine patients, giving a frequency of 15%.

Murphy and Parameswaran (18), Ramana and Chaudhury (19), Mohager et al. (20), and Eltaib et al. (3) reported that *M. catarrhalis* was isolated and identified in 1%, 3.75% 1%, and 8.9% of the patients, respectively. The reason for the difference in the prevalence of infection can be due to the different prevalence of the bacterium in different geographical areas, which has been well proven.

The colonization process of this bacterium in the respiratory tract without clinical symptoms until the development of a symptomatic disease is still not properly cleared. However, a number of bacterial factors that contribute to the pathogenesis have been identified. Some isolated strains have all of these factors. In contrast, some strains lack some of these factors. It is not clear, therefore, whether the expression of these factors would be the main cause of the disease. Environmental and host factors also play an important role in the progression of *M. catarrhalis* infection (21, 22).

The results showed that the frequency of the majority of virulence genes in the isolates of the control group was lower than that of the patients with respiratory infections, which can confirm that in addition to non-pathogenic strains that are lower in the respiratory tract of healthy people, there are some strains that acquire pathogenicity through the acquisition of pathogenic genes.

In a study conducted by Verhaegh et al. (9) to determine the frequency of virulence genes in *M. catarrhalis* isolated from children and adult with respiratory diseases, the prevalence of *mcaP* gene in 99% of the strains and the *ompJ* and *ompCD* genes in 100% of the isolates was observed, which is similar to the results of this study.

In another study conducted by Mollenkvist et al. (23) on the frequency of *MID/Hag* gene, it was shown that all strains (100%) isolated from various clinical conditions carried this gene. In another study conducted by Verhaegh et al. (7) on the genetic diversity of the strains of *M. catarrhalis* in 2011, from among 102 isolated strains, the presence of *MID/Hag* gene was confirmed in 85 strains, giving a frequency of 83%.

The majority of the studies have shown that all isolated strains have the *copB* gene. For example, Bootsma et al. (24) and Verhaegh et al. (9) by analyzing *M. catarrhalis* genes via molecular techniques reported a frequency of 100%. However, in studies conducted by Mitov et al. (25) and Liu et al. (26) on the distribution of pathogens responsible for the pathogenesis of *M. catarrhalis*, the results showed that this gene was present in 50% to 55% of the samples. The reason for this difference is not well understood; however, it can reflect the effect of different geographical regions in this

context.

One of the other virulence factors of this bacterium is the *uspA* membrane proteins; owing to the presence of this factor, the bacterium can attach to the extracellular matrix of the opioid cells to facilitate the formation of biofilms (27). Several studies in this area have demonstrated a significant relationship between the presence of *uspA1* and the potential for pathogenesis. The PCR results showed that 96.9% and 78.2% of the isolates carried *uspA1* and *uspA2* genes, respectively, which indicates the presence of this gene in isolated strains. The frequency of the outer membrane protein genes *uspA1* and *uspA2* has been reported in different studies. For example, Meier et al. (28) evaluated the frequency of *uspA1* and *uspA2* genes as 99% and 77%, respectively. Also, in another study by Verhaegh et al. (7) via genotyping of *M. catarrhalis*, children and adolescents with upper respiratory tract infections were identified to have a higher frequency for *uspA1* gene (99%) compared to *uspA2* (76%). In various studies conducted in different parts of the world, the high presence of *uspA1* and *uspA2* genes was reported in children with middle ear infection (29, 30), patients with pneumonia (31), and patients with COPD (29, 32).

Overall, our findings verified the existence of *M. catarrhalis* in patients with respiratory diseases. The PCR results showed that almost all of the isolates possessed virulence genes. Therefore, the rapid identification and successful treatment can play an important role in preventing their spread.

Footnotes

Conflict of Interest: No conflict of interest is reported.

Ethical Considerations: The study was approved by the National Ethics Committee for Biomedical Research (Code of Ethics: IR.IAU.TON.REC.1397.022), and written consent was obtained from all participants.

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