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Isolation, identification, and antibacterial susceptibility testing of Moraxella catarrhalis isolated from the respiratory system of patients in northern Iran

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ABSTRACT

Background and Objectives: Moraxella catarrhalis is considered as an emerging pathogen and a new nosocomial infection agent. This study was conducted to isolate and identify M. catarrhalis from clinical samples (respiratory tracts) and assess them for antimicrobial susceptibility patterns.

Methods: In total, 280 samples were collected from patients with respiratory tract infection, and 120 samples were obtained from healthy individuals in the control group. The isolates were identified by phenotyping and genotyping methods, and their antibiotic susceptibility was evaluated using disk diffusion methods. The presence of β-lactamase and efflux pump activity were specified via phenotypic methods. Finally, Bro and acrA genes in the isolates were detected by PCR technique.

Results: The frequency of this bacterium was 9.64% (27 out of 280) in patients with respiratory tract infection and 4.16% (5 out of 120) in the control group. Although the isolates were resistant to penicillin, they had various responses against other antibiotics. The results obtained from molecular method showed that 90.6% and 84.3% of the isolates possessed Bro and acrA genes, respectively. There was a significant relationship ($P<0.05$) between the presence of Bro and acrA genes and antibacterial resistance to ampicillin, amoxicillin, cefazolin, cefuroxime, and chloramphenicol.

Conclusion: Our findings confirmed the existence of M. catarrhalis in patients with respiratory diseases and the high prevalence of antibiotic resistant genes in M. catarrhalis isolates. Therefore, timely diagnosis and successful treatment can play important roles in preventing their spread.

Keywords: Moraxella Catarrhalis, Respiratory Tract Infection, Drug Resistance, B-Lactamase

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INTRODUCTION

Moraxella catarrhalis is an important pathogen and a common cause of otitis media, pneumonia, sinusitis, and conjunctivitis in infants, children, and the elderly. In adults, *M. catarrhalis* leads to the formation of chronic obstructive pulmonary disease (COPD) and pneumonia (1-4). This bacterium further contributes to the formation of respiratory tract infections with other pathogens such as *Streptococcus pneumonia* and *Haemophilus influenza* (5-7). To detect this important infection agent, standard culture techniques and phenotypic tests can be employed. However, the cultivation of pharynx, sputum, sinus, and ear discharge is not routinely performed in medical diagnostic laboratories; therefore, the treatment of upper and lower respiratory tract infections is completely experimental and targeted against three main pathogens, namely *M. catarrhalis*, *H. influenzae*, and *S. pneumonie* (8). The response to standard antibiotic treatments has changed, and the prevalence of antibiotic resistance has reached a dangerous level in many treatment centers (9). Different methods make use of bacteria to protect the harmful effects of antibiotics. One of the most important mechanisms utilized in Gram-negative bacteria against β-lactam antibiotics is the production of β-lactamase enzymes which deactivate these antibiotics through the hydrolysis of the β-lactam ring (10). Because most of the isolated *M. catarrhalis* strains are capable of generating β-lactamase owing to the presence of the *Bro* gene, the bacteria are resistant to β-lactam antibiotics such as penicillin. On the other hand, studies have shown that the production of β-lactamases by *M. catarrhalis* has a modest effect on antibiotic susceptibility to other β-lactams and cephalosporins (11). β-lactams are among the most widely used antibiotics in the world; therefore, over the past two decades, creating resistance to these antibiotics has become a major medical challenge (12). Another antimicrobial resistance mechanism in *M. catarrhalis* is the presence of exiting pump. The efflux system is a highly important mechanism for antibiotic resistance, because it protects bacteria against different antibiotic classes such as β-lactams, quinolones, and aminoglycosides (13). There is not sufficient information concerning isolation, identification, antibiotic susceptibility, and the presence of antibiotic resistance genes in *M. catarrhalis* strains in patients with respiratory disease in northern Iran; accordingly, present study aimed to diagnose and appropriately treat the infections originating from this bacterium.

MATERIALS AND METHODS

In this descriptive cross-sectional study, 400 clinical samples, including pharynx's swab (n= 92), secretions of sinus (n= 85), ear discharge (n= 43), pulmonary secretions (n= 60), and pharynx's swab of healthy individuals as control group (n=120) were collected from April 2016 to March 2018. The patients were selected from among those referring to the infectious wards of the public hospitals in the cities of western Mazandaran province in the north of Iran (Noshahr, Tonekabon, and Ramsar) following diagnosis by specialists. The study was approved by the National Ethics Committee for Biomedical Research (Code of Ethics: IR.IAU.TON.REC.1397.022), and written informed consent was obtained from all participants. The samples were transferred to laboratory and subjected to microbiological analysis. To perform microbiological analysis, each sample was cultivated on the blood agar (Merck-Germany) and incubated at 37˚C. After 48 h, all suspected colonies were picked up and phenotypically identified using Gram stain (Zistroyesh-Iran), catalase, oxidase, DNase, and nitrate reduction tests, and fermentation of glucose, sucrose, and lactose (Merck-Germany). To verify the presence of *M. catarrhalis* colonies, the hockey puck test was applied with a wooden stick to push the colonies across the plate.

In this study, the antimicrobial susceptibility of *M. catarrhalis* isolates was determined by disk diffusion test. To carry out the test, each isolate was added into the sterile phosphate buffer saline, and its turbidity was adjusted to 0.5 McFarland tube (1.5×10^8 cells $^{mL-1}$). Afterwards, 100 µl of the suspension was fully streaked on the Mueller-Hinton agar medium (Merck-Germany) and antibiotic discs (Padtan Teb-Iran): penicillin (10 µg), ampicillin (10 µg), amoxycillin (25 µg), cefazolin (30 µg), cefuroxime (30 µg), cefepime (30 µg), ceftriaxone (30 µg), clindamycin (2 µg), co-amoxyclav (20 µg), trimethoprim sulfamethoxazole (1.2 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tetracyclines (30

µg), azithromycin (15 µg), chloramphenicol (30 µg), and erythromycin (15 µg) were placed on the medium and the plates were incubated at 37°C for 48 h. The disc strengths and the zone size were interpreted in accordance with Clinical Laboratory Standard Institute (CLSI 2017) guidelines (2, 14).

The presence of β-lactamase enzyme and Extended-Spectrum β-Lactamases (ESBL) enzyme in antibiotic resistant isolates was determined through the use of the nitrosafin (Sigma-Aldrich-USA) and amoxicillin/clavulanic acid discs (Padtan Teb-Iran), respectively (15). To investigate the presence of efflux pump in the isolates of *M. catarrhalis*, a phenylalanine arginine beta naphtyl amide (Merck-Germany) was used as chemical inhibitor. The cultures were placed under a temperature of 37°C for 24 hours; the growth inhibitory area was then measured using a millimeter ruler and reported as sensitive, resistant or semi-sensitive based on the CLSI 2017 guidelines (16). The molecular identification was carried out by DNA extraction of *M. catarrhalis* using a DNA extraction kit (Isogene- Russian). The purity of the extracted DNA was assessed based on the absorbance of the extracted DNA at 260 and 280 nm wavelengths using bio photometer (Eppendorf-Germany).

Specific primers generated by TAG Copenhagen (Denmark) were employed to amplify the *16SrRNA*, *BRO*, and *acrA* genes. Table 1 shows the primers utilized in the present study.

To conduct 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 × PCR buffer (Geneall, korea), 1 µl of 10 pmol of each primers, 1 µl of 10 mM dNTPs (Geneall, korea), 0.5 µl of smart taq DNA polymerase (Geneall, korea), 1 µl of 50 mM MgCl2 (Geneall, korea), and 5 µl of DNA template. PCR amplification conditions on the thermocycler (Biorad-Germany) were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec (*16SrRNA* gene), 55°C for 60 sec (*Bro* gene), 56°C for 60 sec (*acrA* gene), and 72°C for 60 sec, with a final extension at 72°C for 10 min.

An aliquot of all PCR products was run on a 1.5% (w/v) agarose gels (Merck-Germany) with a 100 bp DNA ladder (Fermentas, Germany) and electrophoresed at 75 V for 40 min. The bands were visualized by use of safe stain and photographed by a UV-transilluminator (UV doc, England).

The statistical analysis of the experimental was done via SPSS version19.

Table 1. The specific primers of *16SrRNA, Bro,* and *acrA* genes

Genes	Primer name	5'→3'	PCR product length	References
16SrRNA	*16SrRNA*-F	TTGGCTTGTGCTAAAATATC	140 bp	17
	16SrRNA-R	GTCATCGCTATCATTCACCT		
BRO	BRO-F	CTTGGCGATGTCTACACC	220 bp	18
	BRO-R	AAGTTTGGCATTGACACG		
acrA	acrA-F	CGTGTTGCCGCCATTGAGACTT	520 bp	19
	acrA-R	GGGAACACAGCGCGTAAGGTCA		

RESULTS

Of the 400 samples, 32 samples were positive for *M. catarrhalis* (8%). Strains were identified using *16SrRNA* gene amplification by PCR technique. The comparison of phenotyping and genotyping identification of the isolates showed that all molecular identifications confirmed our phenotyping identification. The occurrence frequency of this bacterium in the patients with pharyngitis, sinusitis, otitis media, and respiratory failure was 5.43%, 8.24%, 13.95% and 15%, respectively. However, *M. catarrhalis* was isolated from five healthy individuals with a frequency of 4.16%.

Therefore, this bacterium occurred relatively more in patients with respiratory failure. The results obtained from antibiotic susceptibility of the isolates revealed that all isolates were resistant to penicillin (100%), but there were various responses to other antibiotics. The phenotypic examination revealed that all

isolates (100%) had β-lactamase enzymes.

The phenotypic analysis showed that all isolates had extended spectrum beta-lactamases. Furthermore, the phenotypic examination results of efflux pump in the isolates showed that after adding the chemical inhibitor to the environment, the antibacterial activity of various antibiotic groups (penicillins, cephalosporins, phenicols, and tetracyclines) increased.

The obtained data showed that although penicillin resistance did not change after adding the chemical inhibitor, it could indicate another resistance mechanism for this antibiotic; however, there was an increase in the resistance to ampicillin, amoxicillin, cefazolin, cefroxime, chloramphenicol, and amoxicillin-clavulanic acid. Moreover, after performing this test, the amount of resistance to ceftriaxone, cefepim, and tetracycline reached zero (Table 2).

The results showed that the resistance of the above antibiotics to *M. catarrhalis* isolates was dependent on efflux pump because the removal of the efflux pump reduced the diameter of the inhibition zone in this group of antibiotics.

Data presented in Table 2 are the average of three replicates and the values are the total percentage, (n = 32 cases)

Based on the results, 29 isolates possessed *Bro* genes (90.6%) while 27 strains had *acrA* genes (84.3%).

Table 3 shows the association between the *Bro* gene and the resistance of isolates to various antibiotics. As shown, there was a significant relationship between this gene and resistance to ampicillin, amoxicillin, cefazolin, and cefuroxime ($P<0.05$).

Table 4 shows the relationship between the *acrA* gene in *M. catarrhalis* isolates and various antibiotics. As observed, there was a significant relationship between this gene and resistance to ampicillin, amoxicillin, cefazolin, cefroxime, and chloramphenicol ($P<0.05$).

Table 2. Antibiotic resistance patterns of *M. catarrhalis* against different antibiotics based on the CLSI 2017

Antibiotic class	Antibiotic names	With no inhibitors			With inhibitors		
		S	I	R	S	I	R
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
penicillins	penicillin	0 (0%)	0 (0%)	32 (100%)	0 (0%)	0 (0%)	**32 (100%)**
	ampicillin	2 (6.2%)	3 (9.4%)	27 (84.4%)	11 (34.4%)	6 (18.7%)	**15 (46.9%)**
	amoxicillin	3 (9.4%)	3 (9.4%)	26 (81.2%)	14 (43.8%)	5 (15.6%)	**13 (40.6%)**
β-Lactam Inhibitor	amoxicillin-clavulanic acid	28 (87.5%)	2 (6.2%)	2 (6.2%)	30 (93.8%)	1 (3.1%)	**1 (3.1%)**
non-extended spectrum cephalosporins	cefazolin	1 (3.1%)	3 (9.4%)	28 (87.5%)	1 (3.1%)	10 (31.2%)	**21 (65.7%)**
	cefuroxime	1 (3.1%)	4 (12.5%)	27 (84.4%)	9 (28.1%)	6 (18.8%)	**17 (53.1%)**
extended spectrum cephalosporins	ceftriaxone	29 (90.6%)	1 (3.1%)	2 (6.2%)	32 (100%)	0 (0%)	**0 (0%)**
	cefepime	28 (87.5%)	3 (9.4%)	1 (3.1%)	32 (100%)	0 (0%)	**0 (0%)**
aminoglycosides	gentamycin	32 (100%)	0 (0%)	0 (0%)	32 (100%)	0 (0%)	**0 (0%)**
quinolones	ciprofloxacin	32 (100%)	0 (0%)	0 (0%)	32 (100%)	0 (0%)	**0 (0%)**
folate pathway inhibitors	trimethoprim/ sulfamethoxazole	27 (84.4%)	2 (6.2%)	3 (9.4%)	27 (84.4%)	2 (6.2%)	**3 (9.4%)**
lincosamides	clindamycin	30 (93.8%)	2 (6.2%)	0 (0%)	30 (93.8%)	2 (6.2%)	**0 (0%)**
macrolides	azithromycin	29 (90.6%)	3 (9.4%)	0 (0%)	29 (90.6%)	3 (9.4%)	**0 (0%)**
	erythromycin	26 (81.3%)	4 (12.5%)	2 (6.2%)	26 (81.3%)	4 (12.5%)	**2 (6.2%)**
phenicols	chloramphenicol	17 (53.2%)	2 (6.2%)	13 (40.6%)	27 (84.4%)	2 (6.2%)	**3 (9.4%)**
tetracyclines	tetracycline	29 (90.6%)	2 (6.2%)	1 (3.1%)	32 (100%)	0 (0%)	**0 (0%)**

S: sensitive, I: Intermediate, R: resistance

Table 3. The relationship between the presence of *Bro* gene and resistance to different antibiotics in *M. catarrhalis* isolates

Antimicrobial agents	***Bro +***			***Bro -***			**P- value**
	S (%)	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	
penicillin	0 (-)	0 (-)	29 (100)	0 (-)	0 (-)	3 (100)	---
ampicillin	1 (3.4)	1 (3.4)	27 (93.1)	1 (33.3)	2 (66.7)	0 (-)	**0.003**
amoxicillin	1 (3.4)	2 (6.9)	26 (89.7)	2 (66.7)	1 (33.3)	0 (-)	**0.005**
amoxicillin-clavulanic acid	25 (86.2)	2 (6.9)	2 (6.9)	3 (100)	0 (-)	0 (-)	**0.653**
cefazolin	0 (-)	1 (3.4)	28 (96.6)	1 (33.3)	2 (66.7)	0 (-)	**0.001**
cefuroxime	0 (-)	2 (6.9)	27 (93.1)	1 (33.3)	2 (66.7)	0 (-)	**0.002**
ceftriaxone	26 (89.7)	1 (3.4)	2 (6.9)	3 (100)	0 (-)	0 (-)	**0.728**
cefepime	25 (86.2)	3 (10.3)	1 (3.4)	3 (100)	0 (-)	0 (-)	**0.653**
gentamycin	29 (100)	0 (-)	0 (-)	3 (100)	0 (-)	0 (-)	---
ciprofloxacin	29 (100)	0 (-)	0 (-)	3 (100)	0 (-)	0 (-)	---
trimethoprim sulfamethoxazole	24 (82.8)	2 (6.9)	3 (10.3)	3 (100)	0 (-)	0 (-)	**0.584**
clindamycin	27 (93.1)	2 (6.9)	0 (-)	3 (100)	0 (-)	0 (-)	**0.819**
azithromycin	27 (93.1)	2 (6.9)	0 (-)	2 (66.7)	1 (33.3)	0 (-)	**0.263**
erythromycin	23 (79.3)	4 (13.8)	2 (6.9)	3 (100)	0 (-)	0 (-)	**0.530**
chloramphenicol	15 (51.7)	1 (3.4)	13 (44.9)	2 (66.7)	1 (33.3)	0 (-)	**0.086**
tetracycline	26 (89.7)	2 (6.9)	1 (3.4)	3 (100)	0 (-)	0 (-)	**0.728**

Table 4. The relationship between the presence of *acrA* gene and resistance to different antibiotics in *M. catarrhalis* isolates

Antimicrobial agents	**Efflux +**			**Efflux -**			**P- value**
	S (%)	*I (%)*	*R (%)*	*S (%)*	*I (%)*	*R (%)*	
penicillin	0 (-)	0 (-)	27 (100)	0 (-)	0 (-)	5 (100)	---
ampicillin	0 (-)	1 (3.7)	26 (96.3)	2 (40)	2 (40)	1 (20)	**0.001**
amoxicillin	0 (-)	1 (3.7)	26 (96.3)	3 (60)	2 (40)	0 (-)	**0.001**
amoxicillin-clavulanic acid	23 (85.2)	2 (7.4)	2 (7.4)	5 (100)	0 (-)	0 (-)	**0.494**
cefazolin	0 (-)	1 (3.7)	26 (96.3)	1 (20)	2 (40)	2 (40)	**0.002**
cefuroxime	1 (3.7)	0 (-)	26 (96.3)	0 (-)	4 (80)	1 (20)	**0.010**
ceftriaxone	24 (88.9)	1 (3.7)	2 (7.4)	5 (100)	0 (-)	0 (-)	**0.583**
cefepime	23 (85.2)	3 (11.1)	1 (3.7)	5 (100)	0 (-)	0 (-)	**0.494**
gentamycin	27 (100)	0 (-)	0 (-)	5 (100)	0 (-)	0 (-)	---
ciprofloxacin	27 (100)	0 (-)	0 (-)	5 (100)	0 (-)	0 (-)	---
trimethoprim sulfamethoxazole	22 (81.5)	2 (7.4)	3 (11.1)	5 (100)	0 (-)	0 (-)	**0.408**
clindamycin	25 (92.6)	2 (7.4)	0 (-)	5 (100)	0 (-)	0 (-)	**0.708**
azithromycin	26 (96.3)	1 (3.7)	0 (-)	3 (60)	2 (40)	0 (-)	**0.056**
erythromycin	22 (81.5)	3 (11.1)	2 (7.4)	4 (80)	1 (20)	0 (-)	**0.623**
Chloramphenicol	12 (44.4)	2 (7.4)	13 (48.1)	5 (100)	0 (-)	0 (-)	**0.028**
Tetracycline	24 (88.9)	2 (7.4)	1 (3.7)	5 (100)	0 (-)	0 (-)	**0.583**

DISCUSSION

M. catarrhalis is the fifth most prevalent agent of respiratory tract infection following *H. influenza*, *S. pneumonia*, *Klebsiella pneumonia*, and *Pseudomonas aeroginosa* (20-21). This bacterium is able to attach to cellular and mucous surfaces of the respiratory tract, leading to the formation of a biofilm (22-23). Several factors, including age, living conditions, health, environmental agents, and genetics play a role in *M. catarrhalis* infection (24).

In the present study, *M. catarrhalis* was isolated from 32 (8%) samples, a finding similar to the results of khoramrooz et al.(9.5%) and different from the results of Farajzadeh Sheikh et al.(3.6% %) and Sheikhi et al., (42.7%%) in other parts of Iran; This difference is probably attributed to the different sampling times and geographical locations (25-27).

Similar results have been reported in other parts of the world. For instance, the prevalence of *M. catarrhalis* was reported by Tamang *et al*.(6.9%), Osagie *et al*.(4.75%), Mazin *et al*.(3.75%), Elrhman *et al*., (3.75%), and Lu (11%) (2017) (20, 28-31). The highest isolation rate of this bacterium belonged to patients with respiratory failure, otitis media,

sinusitis, and pharyngitis. Of note, *M. catarrhalis* was isolated and identified with a lower prevalence in the control group.

The rapid increase in the antimicrobial resistance to *M. catarrhalis* has caused many concerns in developed and developing countries, hence the need for antibiogram testing to prevent treatment failure and control the infection (13). So far, many studies have been conducted on the drug resistance pattern of *M. catarrhis* isolated from clinical specimens.

The results of Kirby-Bauer test revealed the antimicrobial susceptibility pattern of *M. catarrhalis* isolates against different antibiotics, with the highest resistance to β-lactam antibiotics. The results of the current research are consistent with Krishna et al., Elrhman et al., Hsu et al., Ramadan et al., and Funaki et al. who also reported high resistance to penicillin and cephalosporin antibiotics (2, 30, 32-34).

On the contrary, the isolates were 100% sensitive to ciprofloxacin and gentamicin antibiotics. Therefore, they can be suitable antibiotics for the treatment of infections caused by *M. catarrhalis* isolated from the patients studied in this study. Sensitivity to gentamicin and ciprofloxacin was further delineated by Krishna et al., Gergova et al., and Akinjogunla and Eghafonano (2, 35-36).

Today, resistance mechanisms in bacteria occur in different ways, one of which is the production of β-lactamase enzyme (37). In the present study, phenotypic and PCR techniques were employed to specify the β-lactamase activity and presence of the *Bro* gene. Although β-lactamase activity was observed in all isolates, the presence of *Bro* gene in the isolates of *M. catarrhalis* by specific primers revealed that the gene existed in 29 isolates with a frequency of 90.6%.

In a study conducted by Kadry et al., Mohager et al., and Shi et al. on the molecular detection of *Bro* gene from *M. catarrhalis*, 87%, 73.3%, and 99.4% of the strains possessed *Bro* gene and were fully resistant against penicillin, ampicillin, and cefalutin (38-40).

Another antimicrobial resistance mechanism in *M. catarrhalis* is the presence of efflux pump. Efflux pumps are able to remove a wide range of antimicrobial compounds from the cell (13). In the present study, 27 (3.84%) isolates had *acrA* gene. Moreover, resistance to penicillins and other antibiotics such as aminoglycosides, tetracyclines, and chloramphenicles can be due to the presence of a perfusion pump. Therefore, according to our results, azithromycin, cefotaxime, ceftriaxone, ciprofloxacin, tetracycline, trimethoprim sulfumethoxazole, and gentamicin are the only antibiotic alternatives for the treatment of *M. catarrhalis* infections.

CONCLUSION

M. catarrhalis was present in the samples of patients with respiratory infections. The high resistance incidence of most β-lactam antibiotics in the present study can be considered as a warning signal in the clinics. The presence of resistant genes against β-lactam antibiotics and efflux pump has been proven in most isolates. Resistance to various types of antibiotics is required in the treatment of *M. catarrhalis* infections, hence the necessity of conducting further research and reducing the use of antibiotics.

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CONFLICTS OF INTEREST

None to declare.

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