



ANTIMICROBIAL RESISTANCE OF BULGARIAN ISOLATES *MORAXELLA CATARRHALIS* DURING THE PERIOD 1999-2018

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SUMMARY

The purpose of this study was to assess the levels of antimicrobial susceptibility during a period of the last 20 years among *Moraxella catarrhalis* isolates and to evaluate their resistance mechanisms.

Material/Methods: A total of 618 Bulgarian clinical significant isolates *M. catarrhalis* was collected during the period 1999–2018 from patients to assess the current levels of antimicrobial susceptibility and to evaluate the beta-lactam resistance mechanisms. The minimum inhibitory concentrations (MICs) by E-test were determined against: penicillin, ampicillin, amoxicillin+clavulanic acid, cefalexin, cefuroxime, cefixime, cefotaxime, levofloxacin, azithromycin, clarithromycin, tetracycline, sulfamethoxazole+trimethoprim. PCR examination of genes encoding beta-lactamases was done.

Results: Almost all (98.88%) of the tested strains *M. catarrhalis* showed positive cefinase test and were demonstrated elevated MICs and a lack of susceptibility to penicillin, ampicillin and cefalexin. An increase of resistance to cephalosporin of second and third generations (cefixime) was found. Higher MIC values of cefuroxime and cefixime than these of cefotaxime were detected. PCRs revealed that *bro-1* genes (94.11%) were more frequent than *bro-2*.

Conclusions: The results of examined 20 years period showed that nearly all Bulgarian isolates of *M. catarrhalis* in the last years become resistant to penicillins and first generation cephalosporins via beta-lactamases, encoded predominantly by *bro-1* gene. An increase of the resistance to members of second and third generation (cefixime) cephalosporin's and slower to other antimicrobials was found. The rapid development of resistance in recent years to the most popular antimicrobial agents affects the recommendations regarding the initial approach to therapy.

Keywords: *Moraxella catarrhalis*, beta-lactamases, *bro-1*, *bro-2*, PCR,

INTRODUCTION

Moraxella catarrhalis is an aerobic Gram-negative diplococcus that initially has been considered a harmless commensal microorganism but then has been proved high

virulence of this bacterium. At now, *M. catarrhalis* is one of the three most common causes of childhood otorhinolaryngological infections and chronic cough, as well as exacerbations of chronic obstructive pulmonary disease in adult patients [1-4]. The dynamically increasing incidence of respiratory tract infections caused by this microorganism over the past 15 - 20 years is due to the rapid selection of resistance to the most used beta-lactams and the lack of vaccine yet [5-8]. The extracellular β -lactamase production has an indirect role in the pathogenesis of infection by inactivation of penicillins or cephalosporins suitable for the treatment of other co-existing with *M. catarrhalis* susceptible pathogens in polymicrobial biofilms [9, 10]. The antibiotic resistance and the biofilm formation by *M. catarrhalis* are a major reason for the ability of bacterial cells to survive and persist *in vivo* in the otorhinolaryngological niche or their spreading in down respiratory tract of patients with chronic obstructive disease (COPD) [11].

The aim of this study was to assess the levels of antimicrobial susceptibility during a period of the last 20 years and to evaluate the resistance mechanisms in *M. catarrhalis* to the problematic antimicrobial agents.

MATERIAL AND METHODS

Bacterial strains and samples

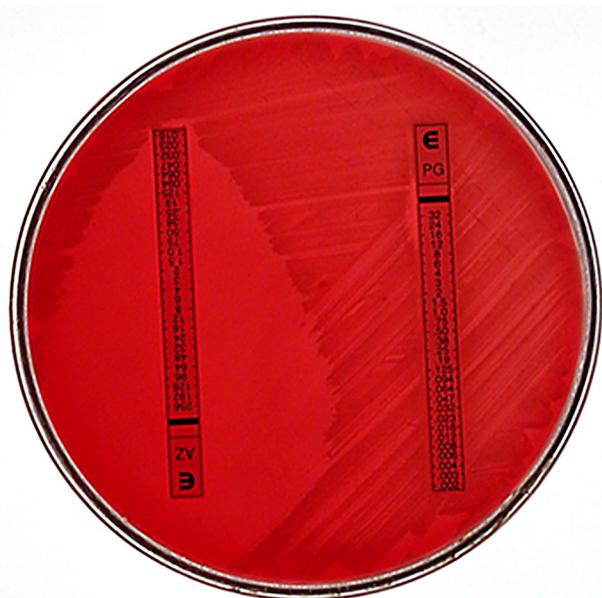
A collection of 618 non-duplicate strains *M. catarrhalis* isolated from Bulgarian patients with signs of respiratory tract infection, living predominantly in Sofia, aged 1–79 years during the period of January 1999 to December 2018 were collected. The clinical samples were: mucosal samples from upper respiratory tract - nasal and pharyngeal swabs, predominantly from children with otorhinological infections or/and chronic cough (N=439); and sputum and bronchial-alveolar lavages (N=179) from adult patients predominantly with COPD. Bacterial cultures were grown routinely for 24-48 h at 37°C on Columbia agar supplemented with 5% sheep blood (Becton Dickinson, Kelberg, Germany) in an atmosphere comprising 95% air and 5% CO₂. Pure bacterial cultures were isolated from suspected colonies with typical cultural phenotype: light gray, not hemolytic, and which can be pushed along the surface of the agar (positive "hockey disk test"). The isolates were identified as *M. catarrhalis* by microscopic morphology -

Gram negative diplococci and positive tests for oxidase, catalase and Indoxylacetate esterase [12]. The suspected pure bacterial cultures additionally identified when need using Crystal NH (Becton Dickinson, Kelberg, Germany). Referent strains of *M. catarrhalis* were used: ATCC 25238; BCCM/LMG 11177, β -lactamase producing, and BCCM/LMG 11178 - β -lactamase non-producing; CIP 103772, with *bro1* genes and CIP 103773 with *bro2*.

Antimicrobial susceptibility testing

Cefinase disks (Becton Dickinson, Kelberg, Germany) were used to investigate β -lactamase production of the examined *M. catarrhalis* strains. Antimicrobial susceptibility testing was conducted using an E-test to estimate the minimum inhibitory concentrations (MICs) value (strips with antibiotics were obtained from Laboratories Pvt. Limited, Mumbai, India) in compliance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [13]. An 0.5 McFarland suspension from pure culture *M. catarrhalis* was used to inoculate an Mueller–Hinton agar plate supplemented with 5% defibrinated horse blood, and Nicotinamide Adenin Dinucleotide 20 mg/L (BIolab Inc., Budapest, Hungary) and E-test strips were placed, and then, the sample was incubated for 18±2 h at 35°C. The breakpoints of MICs were interpreted in accordance with the EUCAST 2019. Antimicrobial susceptibility to twelve antimicrobials (penicillin, ampicillin, amoxicillin+clavulanic acid, cefalexin, cefuroxime, cefixime, cefotaxime, levofloxacin, azithromycin, clarithromycin, tetracycline, sulfamethoxazole+trimethoprim) was evaluated (Fig. 1).

Fig. 1. MICs of penicillin and azythromycin determining by E-test



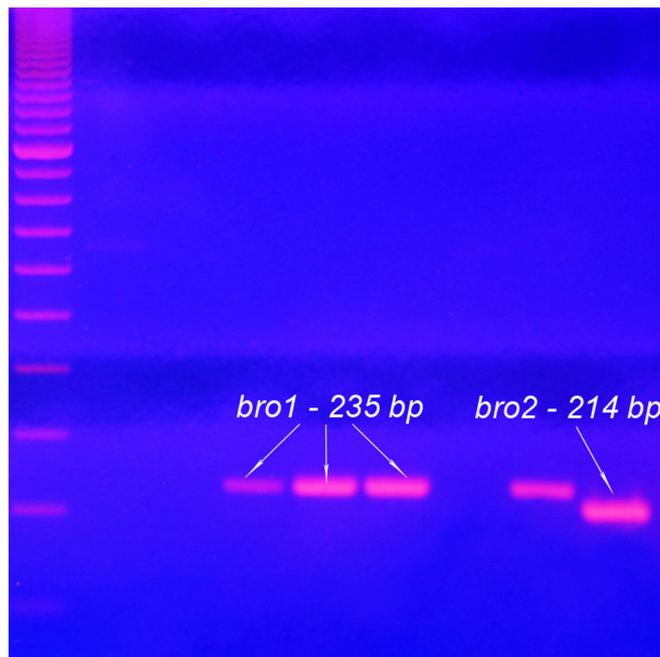
Polymerase chain reaction

For the definitive identification of the examined strains, polymerase chain reaction (PCR) with specific primers (*copB* forward GGCGTGCGTGTTGACCGTTTTG; *copB* reverse GTTTGGCAGGCGATAGGCGACAT) for this bacte-

rial species was performed as previously described by other authors and the amplicons with 564 bp were detected [14].

The genomic DNA was extracted using a DNA sorb-AM nucleic acid extraction kit (AmpliSens, Inter Lab Service, Moscow, Russia) in accordance with the manufacturer's instructions. The pairs of primers for *bro-1* and *bro-2* (5'-TTTGGATTGGGTGAATGAA-3' *bro-1,2* sequences; 5'-TGGGGCTGGGTGATAAATAG-3' *bro-1,2* sequences) were amplified as previously described [4]. The demanded products were the following: *bro1* (235 bp) and *bro2* (214 bp), presented on Fig. 2.

Fig. 2. PCR products of *bro-1* and *bro-2*



STATISTICAL ANALYSIS

Differences were analyzed using unpaired descriptive statistics, Fisher exact test Quick cal. The difference of the susceptibility rates was considered statistically significant at $p < 0.05$.

RESULTS

Ninety eight point nine percent of the isolates recovered during 1999-2008, and almost 100% of those isolated between 2009-2018 were cefinase positive. The determined MICs, MIC90 of 12 antimicrobial agents and their interpretation were showed in Table1. The tested isolates *M. catarrhalis* were demonstrated elevated MICs and a lack of susceptibility to penicillin (Fig. 1), ampicillin and cefalexin. The distribution of antimicrobial susceptibility rates according to the years was shown in Table 2. A decrease of susceptibility rates to cefuroxime and cefixime was found. In the recent period, 2004-2018 has been detected resistance to cefotaxime at low level (Table 2). The detected susceptibility to other antimicrobials such as tetracyclines and sulfamethoxazole+trimethoprim slightly decreased during the period - from 93.4% to 88% (for tetracycline) and from 90.6% to 86.2% (for sulfamethoxazole/trimethoprim). The PCR examination of the genetic ele-

ments encoding beta-lactamases was showed that *bro-1* genes are more frequently detected than *bro-2* (94.11% versus 5.89%), The derived rate of *bro-1* (Fig. 2) for the period 1999-2008 was 90,83% and has become to 98.20%

during 2009-2018. Some isolates were evaluated as with decreased susceptibility to macrolides, especially to clarithromycin (2,44%). Resistant isolates *M. catarrhalis* to quinolones were not detected during the period.

Table 1. MICs of 12 antimicrobial agents to 618 strains *Moraxella catarrhalis*

Antimicrobial agent	MICs		Interpretation	
	Range	90%	S (%)	R (%)
penicillin G	0,002 - > 32	32	0,97	99,03
ampicillin	< 0,016 - 24	8	0,97	99,03
amoxicillin/Clav. acid	< 0,016 - 2	0,25	100	0
cephalexin	0,016 - 32	16	0,97	99,03
cefuroxime	< 0,016 - 8	2	63,92	36,08
cefixime	0,016 - 8	4	69,58	30,42
cefotaxime	< 0,016 - 2	0,25	95,95	4,04
levofloxacin	< 0,016 - 0,5	0,06	100	0
azithromycin	< 0,002 - 1	0,06	99,35	0,65
clarithromycin	0,002 - 4	0,25	97,56	2,44
tetracycline	0,125 - 48	0,5	87,38	12,62
trimethoprim/Sulfamethoxazole	0,005 - 8	0,5	85,76	14,24

Table 2. Susceptibility of 618 *M. catarrhalis* to antimicrobials presented in %.

Antimicrobial agent	1999-2003	2004-2008	2009-2013	2014-2018
penicillin	2,9	1	1,3	0
ampicillin	2,9	1	1,3	0
amoxicillin/clav.acid	100	100	100	100
cefalexin	4,9	1,3	1,6	0
cefuroxime	97.2	86.7	79.1	64.9
cefixime	100	98.9	80.7	62.7
cefotaxime	100	99.4	98.9	97.1
levofloxacin	100	100	100	100
azithromycin	100	100	100	99.7
clarythromylin	100	100	100	98.06
tetracycline	93.4	93.9	90.6	88
sulfomet./trimethoprim	90.6	91.6	88.2	86.2

DISCUSSION

The Bulgarian isolates *M. catarrhalis* have demonstrated the increase of the resistance during the last 20 years toward many beta-lactam antimicrobials due to the production of beta-lactamases, encoded predominantly by *bro-1* gene. The detected MICs in the present study to penicillins and first generation cephalosporins were very high, which is in concordance with positive cefinase test for more than 98% of the isolates. This is proof of the leading role of beta-lactamases. The enzymes of *M. catarrhalis* can be divided into two closely related types, BRO-1 and BRO-2. BRO-1 producing isolates have been re-

ported as more resistant to beta-lactams than BRO-2 strains [8, 15]. The higher resistance rates to penicilins and first generation cephalosporines could be explained with the high percent of *bro-1* producers (>94%). Japan authors had found similar to our rate of *bro-1* -95%, encoding the more active enzyme type and *bro-2* - 5%, respectively [8]. The increased use of beta-lactams has led to a concurrent increase in the number of pathogens with acquired resistance to this drug group. As the link between an antibiotic treatment of the host and microorganisms requires the coordinated activity of many microbial genes, this process leads to new mutations and new changes in

the susceptibility to antimicrobials [15, 16]. Furthermore, at last, fifteen years in Bulgaria, a decrease of susceptibility to some of the second and third generation cephalosporins (cefixime) has been detected. This could be explained most probably with the extension of the spectrum of enzymes, encoded by *bla_{bro-1}*. This phenomenon has been reported from other authors [8, 15-17]. More than 1/3 of tested isolates *M. catarrhalis* during the last five years became resistant to the both of cefuroxime and cefixime. Similar to our results were reported by Saito et., that higher rates of MICs to cefixime and clarithromycin always associated with *bro-1* [16]. The possible extension in the present study has not affected cefotaxime - only about 4% of the isolates were with elevated cefotaxime MICs (2mg/mL). *M. catarrhalis* isolates with elevated ceftriaxone MICs (8 mg/mL) was detected via another Japanese surveillance report [17]. The isolates in the present results were 100% susceptible to amoxicillin/clavulanic acid, which is in concordance with other studies that BRO beta-lactamases were inhibited by clavulanic acid and its combination with amoxicillin demonstrates 100% activity [15-17].

The resistance of *M. catarrhalis* to other antimicrobials still has very small significance for the medical practice in Bulgaria. Resistant isolates to quinolones were not detected among tested strains moraxellae. Nevertheless, some isolates with decreased susceptibility to the other groups of antibiotics have been found in the present work. This is in concordance with other reports [16-19]. The found resistance to tetracycline (resp. doxycycline) and sulfamethoxazole+trimethoprim in the examined isolates *M. catarrhalis* was very low and had no in-

crease during the study period. Most probably those antimicrobials were not used very often in Bulgaria. In contrast to our work, Pakistan authors reported sulfamethoxazole+trimethoprim resistance rate near 60% [18]. Our finding of some difference between MICs of azithromycin and clarithromycin were reported by other authors [8, 20]. The exposure of *M. catarrhalis* to clarithromycin increased the expression of particular components of the efflux pump. Novel mechanisms responsible for resistance to macrolides and quinolones in *M. catarrhalis* were reported and increase of the resistance to these groups antimicrobials in Asia, and Europe were reported [18-21].

CONCLUSIONS

In summary, the results of examined 20 years period showed that nearly all Bulgarian isolates of *M. catarrhalis* in the last years become resistant to penicillins and first generation cephalosporins via the production of beta-lactamases, encoded predominantly by *bro-1* gene. The increased resistance to members of second and third generation cephalosporines (cefixime) and slower to other antimicrobials (tetracycline and sulfamethoxazole/trime-thoprim) was found. Amoxicillin/clavulanic acid was the most effective antimicrobial. The extended spectrum of beta-lactamases appear to have a significant presence among Bulgarian isolates *M. catarrhalis* and can compromise the empiric choice of treatment of infections due to this bacterium. The rapid development of resistance in recent years to the most popular antimicrobial agents affects the recommendations regarding the initial approach to therapy.

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Please cite this article as: Gergova R, Markovska R. Antimicrobial resistance of Bulgarian isolates *Moraxella catarrhalis* during the period 1999-2018. *J of IMAB.* 2020 Apr-Jun;26(2):3208-3212. DOI: <https://doi.org/10.5272/jimab.2020262.3208>

Received: 25/07/2019; Published online: 25/06/2020



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