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FULL PAPER

Tetracycline resistance in *Moraxella catarrhalis* clinical strains isolated in Poland

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SUMMARY

Moraxella catarrhalis is considered an important, exclusively human respiratory tract pathogen which, along with *Streptococcus pneumoniae* and *Haemophilus influenzae*, is classified as one of the most frequent bacterial etiological factors causing upper respiratory tract infections. In this manuscript, we report the existence of five tetracycline-resistant *M. catarrhalis* strains with confirmed presence of tetracycline resistance *tetB* gene. The strains were isolated from children under the age of three with signs of upper respiratory tract infections. Our research also investigated the occurrence of virulence genes in these strains and involved the analysis of drug resistance to five antibiotic groups. It is the first description of clinical strains with confirmed presence of drug resistance *tetB* genes isolated in Europe.

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INTRODUCTION

Moraxella catarrhalis is considered an important, exclusively human respiratory tract pathogen which, along with *Streptococcus pneumoniae* and *Haemophilus influenzae*, is classified as one of the most frequent bacterial etiological factors causing upper respiratory tract infections. The most common clinical forms of infections caused by *M. catarrhalis* include acute otitis media in children and exacerbation of chronic obstructive pulmonary disease in adults (Bernhard *et al.,* 2012, Liu *et al.,* 2016). *M. catarrhalis* is less frequently responsible for causing acute bacterial rhinosinusitis (Abuhammour *et al.,* 1999; Farajzadeh *et al.,* 2016; Santee *et al.,* 2016), laryngitis (Hol *et al.,* 1996), pneumonia (Lutwick & Fernandes, 2006, Sy & Robinson, 2010), and bronchitis (Narang *et al., 2014*). Despite the relatively low virulence of this bacterium, more than 80 invasive infections caused by *M. catarrhalis* have been reported so far. These include, among others, endocarditis (Stefanou *et al.,* 2000; Tanyel *et al.,* 2009; Shahani *et al,* 2015), bacteremia (Abuhammour *et al.,* 1999; Ahmed *et al.,* 2008; Shahani *et al.,* 2015), meningitis (Jin, 2000), ventriculitis (Rotta *et al.,* 1995), conjunctivitis (Paul *et al.,* 2000), orbital cellulitis (Abuhammour *et al.,* 1999), peritonitis (Sadjadi *et al.,* 2012), osteomyelitis (Verjano *et al.,* 2002), and septic arthritis (Olivieri *et al.,* 2004).

M. catarrhalis is usually considered a pathogen exhibiting high susceptibility to antibiotics; however, the therapy should avoid the use of β-lactam antibiotics owing to the extremely common resistance of this bacterium to penicillins resulting from the production of BRO-type β-lactamases, which are specific only for this species. Currently, the production of β-lactamases is exhibited by more than 95% of clinical strains (Flamm *et al.,* 2012; Sheikh *et al.,* 2014; Du *et al.,* 2017; Yamada *et al.,* 2017). Additionally, inherent resistance to vancomycin, clindamycin, and trimethoprim has also been described for this species. Despite that fact, the routinely implemented therapy consisting of a combination of penicillin and β-lactamases inhibitors is usually sufficient. Reports concerning the resistance of *M. catarrhalis* to other antibiotics can rarely be found in the international literature; however, they indicate an alarming, albeit slow, increase of this phenomenon. So far, reports have described, among others, strains resistant to trimethoprim/sulfamethoxazole, levofloxacin, ciprofloxacin, erythromycin, gentamicin, clarithromycin, telithromycin, cefotaxime, or chloramphenicol (Gupta *et al.,* 2011; Flamm *et al.,* 2012; Maraki & Papadakis, 2014; Todberg *et al.,* 2014; Zhang *et al.,* 2016). Strains with decreased susceptibility or resistant to tetracycline have been isolated increasingly more often as well. The number of detected resistant strains is higher in comparison to other antibiotics (Flamm *et al.,* 2012; Hsu *et al.,* 2012; Maraki & Papadakis, 2014). However, the exact mechanism behind the resistance has not been explained so far. Thus, the purpose of this study was to investigate the molecular mechanisms of tetracycline resistance in five tetracycline-resistant *M. catarrhalis* clinical strains isolated in Lublin, Poland, and to analyze those strains for the occurrence of genes encoding virulence factors.

MATERIALS AND METHODS

The bacterial strains were obtained from nasal swabs collected from individuals with signs of upper respiratory tract infection in Lublin in 2017. All patients gave their written informed consent to the use of the isolated strains for the purpose of this study.

The isolation of *M. catarrhalis* was performed at 37°C/24 h on Columbia agar (BD Company) in an atmosphere com-

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prising 95% air and 5% CO_2. The identification of isolated strains was performed by routine bacteriological techniques. Colonies of Gram-negative, catalase- and oxidase-positive diplococci, giving a positive hockey puck test and obtained from pure cultures were also identified with the use of API® NH identification strips (bioMerieux). Additionally, PCR for marker gene *copB* was performed for a definitive identification of the examined strains (denaturation at 94°C/40s, annealing at 55°C/1 min, and extension at 72°C/1 min, each for 35 cycles). *M. catarrhalis* ATCC 25238 strain was used as a positive control.

β-lactamase production was investigated with the use of cefinase disks (MAST ID Graso Biotech, Poland). Disk diffusion method on the MH-F agar in compliance with the EUCAST guidelines (EUCAST Clinical Breakpoint Tables v. 7.1) was performed as a part of the antimicrobial susceptibility testing. Moreover, in order to determine the MIC value, tetracycline susceptibility of the five tetracycline-resistant *M. catarrhalis* strains was analyzed on MH-F agar with the use of Etest (bioMérieux). The antibiotic susceptibility results were interpreted in accordance with the "breakpoint tables for interpretation of minimum inhibitory concentrations (MICs) and zone diameters" specified by EUCAST (EUCAST Clinical Breakpoint Tables v. 7.1).

DNA for the performance of genetic testing was extracted using a commercial kit (QIAamp DNA Mini Kit, Qiagen). 2% agarose gel electrophoresis was used for the visualization of PCR products. They were stained with ethidium bromide. All primer sequences, together with references, used in this study are shown in *Table 1*.

All five strains resistant to tetracycline were examined for the occurrence of six tetracycline resistance genes *tetA*, *tetB*, *tetC*, *tetE*, *tetM*, and *tetO*. For this purpose, separate multiplex PCR reactions were performed for three gene groups: 1 – *tetB* and *tetC*, 2 – *tetA* and *tetE* as well as 3 – *tetM* and *tetO* in compliance with the following protocol: denaturation at 94°C/1min, annealing at 55°C/1 min, and extension at 72°C/1.5 min, each for 35 cycles. Plasmids containing specific genes: *tetA* – pSL18; *tetB* – pRT11; *tetC* – pBR322; *tetE* – pSL1504; *tetM* – pJ13; *tetO* – pUOA1 were used as positive controls.

The studied strains were also analyzed for the occurrence of genes encoding virulence factors: *hag/mid*, *uspA1, uspA2, uspA2H*. The applied PCR reaction conditions were identical to the ones performed for *copB* gene. *M. catarrhalis* ATCC 25238 strain was used as a control.

The studied strains were serotyped with the use of multiplex PCR to detect genes for three types of lipooligosaccharide: *losA, losB, losC*. The conditions of the reaction included denaturation at 94°C/30s, annealing at 53.1°C/1min, and extension at 72°C/4 min, each for 25 cycles. *M. catarrhalis* ATCC 25238 strain was used as a control for serotype A LOS, *M. catarrhalis* CCUG 3292 as a control for serotype B LOS, and *M. catarrhalis* CCUG 26391 as a control for serotype C LOS.

STATISTICA 13.0 software (StatSoft) was used to set up and maintain a database.

RESULTS

All five tetracycline-resistant clinical strains were obtained from children under the age of three with signs of respiratory tract infections (fever, runny nose, redness and swell-

Table 1 - *Sequence of PCR primers.*

Name	*Sequence (5'→ 3')*	*Tm (°C)*	*Amplicon size*	*Reference*
Primers of genes encoding selected virulence factors				
copB forward	GGCGTGCGTGTTGACCGTTTTG	58.6	564bp	Verhaegh *et al.*, 2008
copB reverse	GTTTGGCAGGCGATAGGCGACAT	58.8		
uspA1 forward	CGTTATGCACTAAAAGAGCAGGTC	55.7	247bp	
uspA1 reverse	GCATCTGACCAGCTTAGACCAATC	57.4		
uspA2 forward	GCATCTGCGGATACCAAGTTTG	54.8	289bp	
uspA2/A2H reverse	TTGAGCCATAGCCACCAAGTGC	56.7		
uspA2H forward	CTGAATTTGCCAAAGGTCAT	47.7	310bp	
hag/mid forward	GTCAGCATGTATCATTTTTTAAGG	50.6	738bp	
hag/mid reverse	TGAGCGGTAAATGGTTTAAGTG	51.1		
Primers used for strain serotyping				
losB/C forward	CAAAAGAAGACAAACAAGCAGC	51.1	*losA* - 1.9kb	Edwards *et al.*, 2005
losA forward	ATCCTGCTCCAACTGACTTTC	52.4	*losB* - 3.3kb	
los reverse	CATCAAAAACCCCCCTACC	51.1	*losC* - 4.3kb	
Primers of genes encoding resistance to tetracyclines				
tetA forward	GCTACATCCTGCTTGCCTTC	53.8	210bp	Ng *et al.*, 2001
tetA reverse	CATAGATCGCCGTGAAGAGG	53.8		
tetB forward	TTGGTTAGGGGCAAGTTTTG	49.7	659bp	
tetB reverse	GTAATGGGCCAATAACACCG	51.8		
tetC forward	CTTGAGAGCCTTCAACCCAG	53.8	418bp	
tetC reverse	ATGGTCGTCATCTACCTGCC	53.8		
tetE forward	AAACCACATCCTCCATACGC	51.8	278bp	
tetE reverse	AAATAGGCCACAACCGTC AG	51.8		
tetM forward	GTGGACAAAGGTACAACGAG	51.8	406bp	
tetM reverse	CGGTAAAGTTCGTCACACAC	51.8		
tetO forward	AACTTAGGCATTCTGGCTCAC	52.4	515bp	
tetO reverse	TCCCACTGTTCCATATCGTCA	52.4		

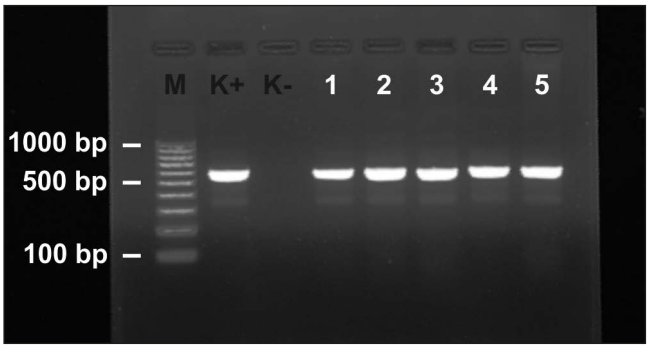

Figure 1 - *PCR amplicons of tetB gene from examined M. catarrhalis strains.*
M - molecular weight marker; K+ positive control: pRT11; K - negative control *M. catarrhalis* ATCC 25238; 1-5 clinical isolates.

ing of the nasal mucous membrane) during routine work conducted in the diagnostic laboratory/diagnostic center at the Department of Medical Microbiology of the Medical University of Lublin. Primary care physicians' referrals were the basis for qualifying patients for the collection of swabs for bacteriological cultures. The study involved the analysis of bacterial isolates with confirmed species affiliation for drug susceptibility regarding the following groups of antimicrobial substances: trimethoprim/sulfamethoxazole (trimethoprim/sulfamethoxazole 1.25-23.75 µg/disk), macrolides (erythromycin 15 µg/disk), fluoroquinolones (nalidixic acid 30 µg/disk, ciprofloxacin 5 µg/disk, moxifloxacin 5 µg/disk, levofloxacin 5 µg/disk), cephalosporins (cefuroxime 30 µg/disk), and penicillins (amoxicillin/clavulanate 20/10 µg/disk). The studied isolates were susceptible to these substances. A disk with 30 µg of antibiotic was used to assess the susceptibility to tetracycline. The range of tetracycline MIC values of the studied strains determined with the use of an Etest was 4-6 mg/l (4 mg/l for the 1st strain, 6 mg/l for the remaining four). The presence of *tetB* gene (*Figure 1*) was detected in all studied strains. The remaining studied genes (*tetA, tetC, tetE, tetM*, and *tetO*) were not present. All five isolates produced β-lactamases, were classified as serotype A LOS, and were characterized by genes encoding virulence factors: *copB, hag/mid, uspA1, uspA2*. None of the strains possessed the gene for UspA2H protein.

DISCUSSION

The studied drug-resistant strains were isolated from individuals with signs of upper respiratory congestion and exhibited almost all of the studied virulence genes. This fact corroborates that *M. catarrhalis* is a pathogen of high clinical importance. Excluding the presence of genes other than *tetB* in the studied isolates constitutes a valuable piece of information concerning this species, which is still poorly characterized in terms of the occurrence of antibiotic resistance mechanisms. The data available in the literature concerning the drug resistance mechanisms of *M. catarrhalis* to tetracyclines is still extremely scarce. Resistance to these antimicrobial substances within *Moraxella* species is observed more often in Asian countries than in Europe or the United States. This may be due to a lack of routine surveillance of the susceptibility to this group of antimicrobials. The bacteriostatic mechanism of tetracyclines involves the blocking of protein biosynthesis through binding and inactivation of the acceptor (A) site in the bacterial ribosome (Brodersen *et al.,* 2000). Owing to a wide activity spectrum (high effectiveness against Gram-positive and Gram-negative bacteria), good tolerance in patients, and low price, tetracyclines are widely and willingly applied in the therapy of bacterial infections. These are also the most commonly used antibiotics in animal husbandry. Tetracycline resistance in bacteria is dependent on the acquisition of genes encoding proteins that condition resistance by one of three mechanisms: ATP-dependent efflux, enzymatic inactivation of the antibiotic, or ribosomal protection (Chopra & Roberts, 2001). Nearly 60 classes of tetracycline resistance genes occurring in more than 530 bacterial species have been described thus far (Liu & Pop, 2009; Warburton *et al.,* 2016).
M. catarrhalis strains collected in this work were analyzed for the occurrence of 6 resistance genes: *tetA*, *tetB*, *tetC*, *tetE*, *tetM*, and *tetO.* The *tetA*, *tetB*, *tetC*, and *tetE* genes encode membrane proteins responsible for the efflux of tetracycline from the cell; *TetB* also conditions the efflux of minocycline. These genes are commonly prevalent in nature (especially *tetB*) and occur only in Gram-negative bacteria. The *tetM* and *tetO* genes encode proteins protecting ribosomes from tetracycline binding, and occur both in Gram-positive and Gram-negative bacteria. Tetracycline resistance genes are most often encoded on mobile genetic elements: *tetA*, *tetB*, *tetC*, and *tetE* genes are most often connected with large conjugative plasmids, *tetM* gene is often connected with large conjugative transposon, which also carries *ermF* gene conditioning resistance to erythromycin, whereas *tetO* gene was detected in both conjugative plasmids and in chromosomes (Chopra & Roberts, 2001). The presence of *tetB* gene was described for various species of Gram-negative bacteria, including those co-existing with *M. catarrhalis* in the upper respiratory tract and occupying the same niche: *H. influenzae*, *H. parainfluenzae*, *Actinobacillus actinomycetemcomitans*, or *Treponema denticola*. It was demonstrated that *tetB* gene in *Actinobacillus* and *Haemophilus* is encoded on the mobile plasmid and may be transferred between those species (Marshal *et al.,* 1984; Roberts, 1989; Roe *et al.,* 1995; Chopra & Roberts, 2001). The acquisition of tetracycline resistance genes by *M. catarrhalis* through horizontal gene transfer from other species or strains seems quite likely, since this mechanism is widely prevalent in other Gram-negative bacteria. However, the presence of *tetB* gene has been described in only four *M. catarrhalis* strains so far. In two strains, the gene was encoded chromosomally and was most probably acquired through transformation (Roberts, 1989; Roberts *et al.,* 1990; Roberts *et al.,* 1991). Apart from these findings, there is no other data concerning the resistance mechanisms of *M. catarrhalis* to tetracyclines in the literature. Since the *M. catarrhalis* genome does not undergo frequent changes and the cells of these bacteria infrequently possess plasmids, it is assumed that the main genetic changes within this species occur through transformation (Davie *et al.,* 2011). Therefore, it can be surmised that the *tetB* genes detected in this work are also located chromosomally, despite the fact that this hypothesis requires further research that is currently in progress. Nevertheless, the authors of this work have decided to publish their results at the current development stage because they provide new and highly significant data concerning *M. catarrhalis*, which remains an insufficiently described pathogen. To our knowledge,

this work is the first description of tetracycline-resistant clinical strains of *M. catarrhalis* with described tetracycline resistance genes isolated in Poland and in Europe. We believe that every effort should be made to include drug susceptibility testing of *M. catarrhalis* to tetracyclines in the standard protocol for microbiological diagnostics of upper respiratory tract infections. It will enable routine surveillance of the increase of drug resistance level of this important pathogen.

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Compliance with Ethical Standards
This research fully complies with the Ethical Standards applicable for this journal and the relevant national and international ethics-related rules and professional codes of conduct. The research protocol was approved by the Ethics Committee of the Medical University of Lublin (number KE-0254/155/2015).

Authors' Disclosure Statement
No competing financial interests exist and all authors report no conflicts of interest in this work.

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