Journal of Microbiology, Immunology and Infection (2017) 50, 386–389

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BRIEF COMMUNICATION

Antimicrobial susceptibility to β-lactam antibiotics and production of BRO β-lactamase in clinical isolates of *Moraxella catarrhalis* from a Japanese hospital

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Received 31 December 2015; received in revised form 21 July 2016; accepted 8 August 2016
Available online 18 December 2016

KEYWORDS
antimicrobial susceptibility;
β-lactam;
BRO β-lactamase;
ceftriaxone;
Moraxella catarrhalis

Abstract We investigated BRO-β-lactamase production of *Moraxella catarrhalis* isolates and its antimicrobial susceptibility to β-lactams. Of the 233 isolates, 232 were BRO producers and 224 were BRO-1 producers. Four isolates exhibited elevated ceftriaxone minimum inhibitory concentration (2 μg/mL) and different pulsed-field gel electrophoresis patterns and we expect this number to increase in the near future.

Introduction

Moraxella catarrhalis is a Gram-negative, aerobic, diplococcal bacterium that is frequently isolated from the postnasal nasopharynx in children, and is one of the most common pathogens responsible for respiratory tract infections, including otitis media, sinusitis, and pneumonia.[1] Although *M. catarrhalis* isolates are generally susceptible to various antibiotics, most clinical isolates are resistance to penicillin due to the production of β-lactamase. BRO β-lactamase-producing *M. catarrhalis* isolates have been reported since the 1970s, and the rate at which β-lactamase-producing clinical *M. catarrhalis* isolates are reported has increased over time. Indeed, several previous reports have demonstrated that >95% of global clinical *M. catarrhalis* isolates produce BRO-β-lactamase.[1,2] At

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http://dx.doi.org/10.1016/j.jmii.2016.08.003

present, two types of BRO enzymes have been characterized: BRO-1 and BRO-2, which are encoded by the *bro-1* and *bro-2* genes, respectively. The two enzymes can be differentiated by the presence of a 21-base-pair (bp) deletion in the promoter region of the *bro-2* gene, compared to that of *bro-1*.[1] Notably, several previous reports indicate that the minimum inhibitory concentrations (MICs) of β-lactam antibiotics for BRO-1 producers are higher than those for BRO-2 producers.[1,2]

β-Lactam antibiotics bind to penicillin-binding proteins and exert antibacterial effects by disrupting the bacterial cell wall composition. Because these broad-spectrum antibiotics are useful against multiple pathogenic bacterial species, they are frequently used to treat respiratory infections caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *M. catarrhalis*. The increased use of β-lactam antibiotics has led to a concurrent increase in the number of pathogens with acquired β-lactam antibiotic resistance. In fact, infectious diseases caused by pathogens exhibiting resistance to β-lactam antibiotics (e.g., *S. pneumoniae* and *H. influenzae*) have become a global problem. However, little attention has been paid to the acquisition of β-lactam antibiotic resistance by clinical *M. catarrhalis* isolates. Although *M. catarrhalis* has recently been described as an important pathogen associated with respiratory infections, antimicrobial susceptibility testing for *M. catarrhalis* has not been consistently performed. Thus, the available information regarding the susceptibility of clinical *M. catarrhalis* isolates to β-lactam antibiotics is insufficient compared to that for other respiratory pathogens in Japan. Therefore, we investigated β-lactam antibiotic susceptibility and BRO β-lactamase production in clinical *M. catarrhalis* isolates.

Methods

Bacterial strains and culture conditions

A total of 233 nonduplicated clinical *M. catarrhalis* isolates (collected from 127 male and 106 female patients) were obtained from nasopharyngeal swabs, sputum, otorrhea, and eye discharge samples between January 2013 and July 2015 at the Tokyo Metropolitan Health and Medical Treatment Corporation Toshima Hospital in Tokyo, Japan. The reference strain ATCC49143 (a BRO-negative strain) was used as a control. All isolates were cultured at 35°C on trypticase soy agar plates containing 5% sheep blood (BD, Tokyo, Japan) in an atmosphere comprised of 95% air and 5% CO_2. Strain identification was confirmed using Gram staining and the ID Test HN-20 Rapid (Nissui, Tokyo, Japan). The isolates were maintained in 10% skimmed milk, and were stored at −80°C.

Detection of β-lactamase production and bro genes

The production of β-lactamase was tested using the nitrocefin test (cefinase disks; BD). Polymerase chain reaction (PCR) amplification of the *bro* genes was performed as previously described.[1] The PCR products were separated via electrophoresis on a 3.0% agarose gel, and were visualized by ethidium bromide staining. The *bro* types were confirmed based on observed differences in PCR product mobility. Partial *bro-1* and *bro-2* products were located at ∼160 bp and 140 bp on the agarose gel, respectively.

Antimicrobial susceptibility testing

The MICs of amoxicillin, amoxicillin−clavulanate, cefaclor, and ceftriaxone were determined using the Etest (Sysmex bioMérieux, Tokyo, Japan) on Mueller−Hinton agar plates containing 5% sheep blood. *Staphylococcus aureus* ATCC29213 was used as a quality control. The breakpoints used to categorize the susceptibility of strains to amoxicillin−clavulanate, cefaclor, and ceftriaxone were defined by the Clinical Laboratory Standards Institute (CLSI) guideline M48-A2.[3] A Mann−Whitney *U* test was used to determine statistical differences in the geometric mean MICs, and $p < 0.05$ was considered statistically significant.

Pulsed-field gel electrophoresis

Isolates were analyzed by pulsed-field gel electrophoresis (PFGE), as previously described.[4] Each DNA preparation was digested with 2.5 U *Spe*I (New England Biolabs, Ipswich, MA, USA) in 300 μL restriction endonuclease buffer overnight at 37°C. The resulting fragments were then separated using the Gene Path system (Bio-Rad Laboratories, Hercules, CA, USA).

Results

The demographics of the *M. catarrhalis* isolates harvested from clinical specimens were as follows: 149 isolates (63.9%) from postnasal drip, 81 (34.8%) from sputum, two (0.9%) from otorrhea, and one (0.4%) from eye discharge. A total of 153 (65.7%) *M. catarrhalis* isolates were recovered from young children (age ≤ 10 years), and 80 (34.3%) were isolated from older children and adults (age > 10 years). Approximately half of the isolates used were colonization isolates.

Among the 233 clinical isolates tested, 232 were characterized as BRO-producing strains (99.6%) via the nitrocefin test. Meanwhile, *bro* gene-specific PCR analysis identified 224 (96.6%) and eight (3.4%) of the isolates as BRO-1 and BRO-2 producers, respectively. In contrast, only one isolate (0.4%) was BRO negative.

The MIC distributions of the four β-lactam antibiotics tested are shown in Figure 1. The distribution for each antibiotic was as follows: 0.032−16 μg/mL for amoxicillin, 0.032−0.5 μg/mL for amoxicillin−clavulanate, 0.5−48 μg/mL for cefaclor, and 0.016−2 μg/mL for ceftriaxone. Among the three β-lactam antibiotics with CLSI-defined breakpoints tested, only that for cefaclor was exceeded (susceptible: ≤8 μg/mL). The rate of resistance to cefaclor was 3.9% (9 of 233 isolates) in this study. The MIC ranges, MIC_{50}, MIC_{90}, and geometric MIC means for the clinical isolates are summarized in Table 1. The geometric mean MICs of amoxicillin, amoxicillin−clavulanate, cefaclor, and ceftriaxone for BRO-1 producers were 3.4-, 1.6-, 2.2-, and 4.8-fold higher than those observed in BRO-2 producers

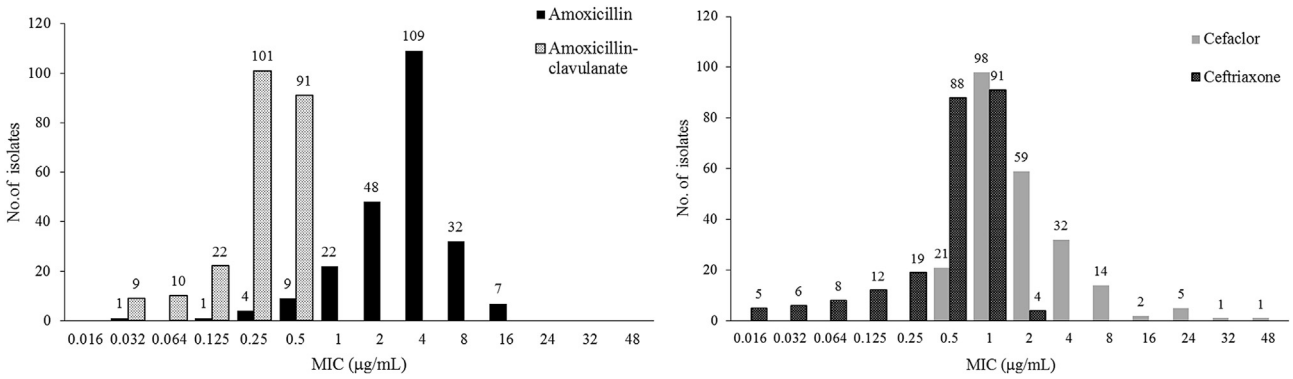

Figure 1. Distribution of the minimum inhibitory concentrations (MICs) of four β-lactam antibiotics for clinical *Moraxella catarrhalis* isolates. These data include all clinical isolates tested (i.e., BRO-1 producers, BRO-2 producers, and the BRO-negative isolate).

Table 1 β-Lactam susceptibility of clinical *Moraxella catarrhalis* isolates.

Antimicrobial agent and phenotype (no. of isolates; isolation rate)	MIC (μg/mL)	
	Range	Geometric mean
Amoxicillin		
BRO negative (1; 0.4%)	0.032	—
BRO-1 producer (224; 96.2%)	0.25—16	3.15*
BRO-2 producer (8; 3.4%)	0.125—4	0.92
Amoxicillin clavulanate		
BRO negative	0.032	—
BRO-1 producer	0.032—0.5	0.28**
BRO-2 producer	0.032—0.5	0.13
Cefaclor		
BRO negative	0.5	—
BRO-1 producer	0.5—48	1.78***
BRO-2 producer	0.5—2	1.09
Ceftriaxone		
BRO negative	0.016	—
BRO-1 producer	0.016—2	0.51***
BRO-2 producer	0.016—0.5	0.11

* $p = 0.002$ versus BRO-2 isolates.
** $p = 0.01$ versus BRO-2 isolates.
*** $p < 0.001$ versus BRO-2 isolates.
MIC = minimum inhibitory concentration.

($p < 0.05$), respectively. Each of the four isolates associated with reduced ceftriaxone MICs (2 μg/mL) exhibited distinct PFGE patterns (data not shown).

Discussion

Acquired resistance to macrolides and fluoroquinolones in Asian *M. catarrhalis* isolates has been reported in recent studies.[5,6] Indeed, Hsu et al[7] reported increasing rates of *M. catarrhalis* resistance to cefaclor, cefuroxime, tetracycline, and trimethoprim—sulfamethoxazole in Taiwan. Based on these reports, it is thought that *M. catarrhalis* clinical isolates in Asia pose a threat due to antimicrobial resistance. Of the numerous antibiotics that are used globally, several β-lactam antibiotics have been used to treat infectious diseases in Japan because of their availability and broad therapeutic applications. However, respiratory pathogens with resistance to β-lactam antibiotics have become a serious problem. Moreover, antimicrobial susceptibility testing for *M. catarrhalis* is lacking in most clinical laboratories in Japan. In this study, we therefore investigated the susceptibility of *M. catarrhalis* clinical isolates to β-lactam antibiotics.

Saito et al[2] reported that there is a high frequency (95%) of BRO-producing *M. catarrhalis* strains in Japan. Furthermore, this group demonstrated that the prevalence of strains encoding the *bro-1* and *bro-2* genes was 95% and 5%, respectively. Consistent with these findings, the prevalence of BRO producers in the current study was markedly high (232 of 233; 99.6%), and 99.6% and 3.4% of these strains encoded *bro-1* and *bro-2*, respectively. However, to the best of our knowledge, the current study detected the highest prevalence of BRO-1 producers to date, highlighting the rapid spread of BRO-1 producers in Japan. The MICs of β-lactam antibiotics for BRO-1 producers were significantly higher than those of BRO-2 producers, which was also similar to previous findings.[2]

Although Bell et al[8] detected BRO-1-producing *M. catarrhalis* isolates that were not susceptible to ceftriaxone (MIC: 4 μg/mL), the percentage of nonsusceptible strains was low (0.6%; 2 of 320). In addition, clinical *M. catarrhalis* isolates with elevated ceftriaxone MICs (8 μg/mL) were confirmed via Japanese surveillance.[9] In this study, the MICs of the majority of the β-lactam antibiotics examined were lower than the determined breakpoints; however, that for cefaclor was exceeded. While the MICs of amoxicillin—clavulanate were low (0.032—0.5 μg/mL), four of 233 clinical isolates (1.7%) exhibited reduced susceptibility to ceftriaxone (MIC: 2 μg/mL). The number of ceftriaxone-resistant *M. catarrhalis* isolates may increase in the near future.

Despite the majority of clinical *M. catarrhalis* isolates being classified as *bro-1* strains, reduced β-lactam antibiotic MICs were observed in this study. Richter et al[10] suggested that alterations in *bro* expression (e.g., promoter-up mutations) might be associated with the diversity of β-lactam antibiotic MICs. In this study, we did not perform BRO genetic analyses, such as sequencing or

quantitative PCR of the *bro* genes, but these analyses should be conducted in future studies. Because the isolates used in this study were primarily colonization isolates, future studies are necessary to examine resistance in *M. catarrhalis* isolated from clinically important infectious diseases.

In summary, we examined the susceptibility to β-lactam antibiotics and the frequency of BRO production in clinical *M. catarrhalis* isolates. The results of our analyses suggest that there has been an increase in the spread of BRO-1 producers in Tokyo. Indeed, the frequency of *M. catarrhalis* isolates with acquired resistance to β-lactam antibiotics is expected to increase in the near future. Therefore, we plan to continue our study of antimicrobial susceptibility in clinical *M. catarrhalis* isolates.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

This study was supported by the Clinical Research Grant of Tokyo Metropolitan Health and Medical Treatment Corporation Toshima Hospital, Tokyo, Japan.

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