ANTIBIOTIC SUSCEPTIBILITIES AND β-LACTAMASE PRODUCTION OF *MORAXELLA CATARRHALIS* ISOLATES FROM ZAGREB, CROATIA

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SUMMARY – *Moraxella catarrhalis*, a commensal of the nasopharynx, has been recognized with increasing frequency as a potential pathogen in respiratory tract infections. The β-lactamase production in *Moraxella catarrhalis*, first described in 1977, has been ever more frequently reported in many countries of the world. However, there have been no such reports from Croatia so far. The aim of this study was to investigate antibiotic susceptibilities and β-lactamase production in *Moraxella catarrhalis* isolates from Croatia. Fifty *Moraxella catarrhalis* strains were collected from various clinical specimens at Zagreb University Children’s Hospital during the 1990-1992 period. Antibiotic susceptibilities to a wide range of antibiotics were determined by the broth microdilution method according to NCCLS. In all strains, β-lactamases were detected by the disk chromogenic substrate (nitrocefin) test. The prevalence of β-lactamase positive strains in the study period was 100%. No resistance to amoxicillin/clavulanate, cephalexin, ceftibuten, tetracycline, erythromycin, azithromycin and chloramphenicol was observed. In all strains, the activity of amoxicillin was strongly enhanced in the presence of clavulanic acid. Older cephalosporins were equally active, however, the third generation cephalosporin ceftibuten showed significantly lower minimal inhibitory concentrations compared with older compounds. Among non β-lactam antibiotics, tetracycline and erithromycin showed similar activity. Azithromycin had a markedly stronger inhibitory activity in comparison with erythromycin and tetracycline. According to our results, amoxicillin combined with clavulanic acid should be the antibiotic of choice for the treatment of infections caused by β-lactamase positive isolates of *Moraxella catarrhalis*. Oral cephalosporins, tetracycline, macrolides or azithromycin could be an option too.

Key words: *Moraxella*-Branhamella catarrhalis – drug effects; Antiinfective agents – pharmacology; Respiratory tract infections – microbiology; Beta-lactamases – biosynthesis; Croatia

Introduction

*Moraxella (M.) catarrhalis*, a commensal of the nasopharynx, has been recognized with increasing frequency as a potential pathogen in respiratory tract infections. It is an important causative agent of lower respiratory tract infections, otitis media and sinusitis. *M. catarrhalis* is fully capable of causing systemic diseases such as septicemia and meningitis in immunocompromised host. These organisms have been transferred from the genus *Neisseria*. The β-lactamase production in *M. catarrhalis*, first described in 1977, has been ever more frequently reported in many countries of the world, e.g., the Netherlands, Sweden, United Kingdom, Finland, Italy, Germany, United States, Canada, Japan, Hong-Kong, South Korea and China. There have been no reports on the frequency of β-lactamase production in *M. catarrhalis* from Croatia so far.

There are three types of β-lactamas described: BRO-1, BRO-2 and BRO-3 with similar substrate profiles, isoelectric focusing patterns and sensitivity to inhibitors. They all are plasmid mediated, hydrolyze carbenicillin,
cefaclor and methicillin (but not cloxacillin) as rapidly as benzylpenicillin, and are strongly susceptible to inhibition by clavulanate. BRO β-lactamases are transferable from one strain to another by transconjugation due to plasmid location of the encoding gene.

BRO β-lactamase positive isolates of *M. catarrhalis* are usually resistant to penicillin, amoxicillin, ampicillin and carbenicillin but are susceptible to a combination of amoxicillin and clavulanic acid, oral and parenteral cephalosporins, carbapenems, macrolides, tetracyclines, chloramphenicol and fluoroquinolones.

β-lactamases of *M. catarrhalis* are produced in small amounts and are located in periplasmic space. The most frequently used test for routine laboratory detection of this enzyme is the chromogenic (nitrocefin) test. Enzymes can also be detected by acidimetric and iodometric procedures, however, they are more laborious and time consuming. The reduced zone diameter around penicillin disk can predict the presence of β-lactamase. Amoxicillin combined with clavulanic acid (coamoxiclav) is the antibiotic of choice for the treatment of infections caused by lactamase positive strains of *M. catarrhalis*.

### Materials and Methods

#### Bacteria

During the 1990-1992 period, 50 penicillin resistant *M. catarrhalis* strains were collected at Zagreb University Children’s Hospital from various clinical specimens (nasopharyngeal swab, middle ear fluid, sputum, bronchoaspirates). The bacteria were identified by conventional biochemical tests.

#### Detection of β-lactamases

β-Lactamases were detected by the commercially available chromogenic cephalosporin disk β-lactamase test containing nitrocefin as substrate (Cefinase disks; BBL Microbiology Systems, Cockeysville, MD). The test was repeated with crude enzyme preparations. Fifty µL of nitrocefin solution (500 mg/L) were mixed with unpurified enzyme sample from each strain. The test was considered positive if the yellow substrate color turned red.

#### Minimal inhibitory concentrations

Minimal inhibitory concentrations (MIC) of ampicillin, amoxicillin, amoxicillin/clavulanic, cephalaxin, cefuroxime, cefadroxil, cefprozil, cefditoren, tetracycline, erythromycin and azithromycin were determined by a twofold microdilution technique using microtiter plates and Mueller-Hinton broth inoculated with 10^5 CFU/ml. The tested range of antibiotic concentrations was 0.015-128 mg/L. Clavulanic acid was added to amoxicillin in a fixed concentration of 4 mg/L. The antibiotic powders were provided by the following manufacturers: ampicillin, amoxicillin, clavulanate, cephalaxin, cefuroxime, tetracycline, erythromycin, azithromycin and chloramphenicol from Pliva, Zagreb, Croatia; cefadroxil and cefprozil from Bristol Myers Squibb, Zagreb, Croatia; and cefditoren and cefditoren from Bristol Myers Squibb, Zagreb, Croatia. The strains were kindly provided by Prof. Ellen Stobbering (Maastricht University Hospital, Maastricht, The Netherlands). The concentrations of antibiotics which inhibited 50% and 90% of the strains (MIC<sub>50</sub> and MIC<sub>90</sub>) were calculated.

#### Characterization of β-lactamases

Strains were grown in Brain-Heart infusion broth at 37 °C with shaking and harvested during late log phase. The cells were pelleted by centrifugation at 4 °C, washed twice in 0.1 M phosphate buffer (pH, 7.0), and resuspended in 1/50 of the original volume. After sonication in ice-bath, the cellular debris were removed by centrifugation at 14 000 rpm for 30 min at 4 °C. The crude cellular extract was stored at -20 °C.

The crude β-lactamase extracts were used to evaluate their ability to break down β-lactam antibiotics (ampicillin, cephalaxin, cefuroxime, cefditoren) by a microbiologic method. Antimicrobial disks were impregnated with crude extract from different isolates. The inhibition zones produced by them and by nonimpregnated disks against *E. coli* ATCC 25922 were compared. Reduction of the inhibition zone with the impregnated disk was considered as evidence for β-lactamase activity against the respective antimicrobial agent.
Results

Detection of β-lactamases

In all strains, β-lactamases were detected with both nitrocefin tests.

Prevalence of β-lactamase positive M. catarrhalis strains

The prevalence of β-lactamase positive M. catarrhalis strains was 100% (50/50).

Antibiotic susceptibilities

Antibiotic susceptibilities are shown in Table 1. MIC ranges for various antibiotics were as follows: ampicillin 1-32 mg/L; amoxicillin 4-32 mg/L; amoxicillin combined with clavulanic acid 0.015-0.5 mg/L; cephalaxin 1-16 mg/L; cefuroxime 0.5-32 mg/L; cefprozil and cefadroxil 0.12-32 mg/L; cefotibutin 0.015-1 mg/L; tetracycline and erythromycin 0.015-0.5 mg/L; azithromycin <0.015-0.25 mg/L; and chloramphenicol 0.06-1 mg/L. The concentrations of particular antibiotics necessary to inhibit 50% of the strains were as follows: azithromycin 0.015 mg/L; erythromycin 0.06 mg/L; tetracycline, amoxicillin+clavulanic acid and cefotibutin 0.12 mg/L; chloramphenicol 0.25 mg/L; cefprozil and cefadroxil 1 mg/L; cefuroxime 2 mg/L; cephalaxin and ampicillin 4 mg/L; and amoxicillin 8 mg/L. The concentrations of particular antibiotics necessary to inhibit 90% of the strains were as follows: azithromycin 0.12 mg/L; erythromycin, tetracycline and amoxicillin+clavulanic acid 0.25 mg/L; cefotibutin and chloramphenicol 1 mg/L; cefadroxil 4 mg/L; cefprozil, cefuroxime, cephalaxin and ampicillin 16 mg/L; and amoxicillin 32 mg/L. Eighty percent (40/50) of the strains were resistant to amoxicillin, 78% (39/50) to ampicillin, 4% (2/50) to cefadroxil, and 2% (1/50) to cefuroxime and cefprozil. No resistance to cephalaxin, cefotibutin, tetracycline, erythromycin, azithromycin and chloramphenicol was observed. In all strains, the activity of azithromycin was strongly enhanced in the presence of clavulanic acid. Older cephalosporins were equally active, however, the third generation cephalosporin cefotibutin displayed significantly lower MICs compared with older compounds. Among non β-lactam antibiotics tetracycline and erythromycin showed similar activity. Azithromycin had a markedly stronger inhibitory activity in comparison with erythromycin and tetracycline.

Characterization of β-lactamases

Crude β-lactamase extracts from M. catarrhalis strains antagonized the activities of disks containing ampicillin (10 µg). The enzymes did not affect the inhibition zones around cephalosporin disks.

Discussion

The prevalence of β-lactamase positive strains (100%) in Croatia is equivalent to that in Japan\textsuperscript{18} and higher than that in the United States (92%)\textsuperscript{15}, Canada (89%)\textsuperscript{45}, Far East

Table 1. Minimal inhibitory concentrations (MIC) of Moraxella catarrhalis; concentrations necessary to inhibit 50% and 90% of isolates and percentage of resistant strains according to NCCLS

<table>
<thead>
<tr>
<th>Antibiotic and resistance breakpoint</th>
<th>MIC range (mg/L)</th>
<th>MIC\textsubscript{50}</th>
<th>MIC\textsubscript{90}</th>
<th>Number and percentage of resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (≥4)</td>
<td>1-32</td>
<td>4</td>
<td>16</td>
<td>39/50 (78%)</td>
</tr>
<tr>
<td>Amoxicillin (≥8)</td>
<td>4-32</td>
<td>8</td>
<td>32</td>
<td>40/50 (80%)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate (≥8/4)</td>
<td>0.015-0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Cephalaxin (≥32)</td>
<td>1-16</td>
<td>4</td>
<td>16</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Cefuroxime (≥32)</td>
<td>0.5-32</td>
<td>2</td>
<td>16</td>
<td>1/50 (2%)</td>
</tr>
<tr>
<td>Cefprozil (≥32)</td>
<td>0.12-32</td>
<td>1</td>
<td>16</td>
<td>1/50 (2%)</td>
</tr>
<tr>
<td>Cefadroxil (≥32)</td>
<td>0.12-32</td>
<td>1</td>
<td>4</td>
<td>2/50 (4%)</td>
</tr>
<tr>
<td>Cefotibutin (≥32)</td>
<td>0.015-1</td>
<td>0.12</td>
<td>1</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Tetracycline (≥28)</td>
<td>0.015-0.5</td>
<td>0.12</td>
<td>0.25</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Erythromycin (≥8)</td>
<td>0.015-0.5</td>
<td>0.06</td>
<td>0.25</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Azithromycin (≥8)</td>
<td>&lt;0.015-0.25</td>
<td>0.015</td>
<td>0.12</td>
<td>0/50 (0%)</td>
</tr>
<tr>
<td>Chloramphenicol (≥8)</td>
<td>0.06-1</td>
<td>0.25</td>
<td>1</td>
<td>0/50 (0%)</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Breakpoints for Haemophilus were used.
(97.6%)\(^{18}\), Italy (84%)\(^{12}\), Finland (96%)\(^{11}\), the Netherlands (39%)\(^{9}\) and United Kingdom (90.8%)\(^{10}\). All strains were found to possess a β-lactamase that was easily detectable due to its extracellular location. Contrary to β-lactamasas from Enterobacteriaceae with M. catarrhalis, there is no need to release the enzymes from the cells by sonication because of the lack of permeability barrier. The enzymes from all strains were strongly inhibited by clavulanate. The wide range of MICs for ampicillin and amoxicillin alone and in combination with clavulanate could be attributed to variable levels of enzyme production by M. catarrhalis strains. The substrate profile of the enzymes was typical for penicillinase type of β-lactamases. They did not hydrolyze significantly cephalosporin substrates although the MICs for cephalosporins are generally lower in β-lactamase lyze significantly cephalosporin substrates although the MICs for cephalosporins are generally lower in β-lactamase negative strains, as reported elsewhere\(^{8,10}\). In our collection, there were no β-lactamase negative strains to compare their MICs with those from β-lactamase positive ones.

The results of the susceptibility tests were in concordance with the results reported by other investigators\(^{9,10,13,17,31}\). The β-lactamase positive strains of M. catarrhalis are generally susceptible to all antibiotics except for unprotected penicillins. In order to prevent the emergence of antibiotic resistant isolates, the rational use of antimicrobials and monitoring of the antibiotic resistance of respiratory pathogens will be required.

According to our results, amoxicillin combined with clavulanic acid should be the antibiotic of choice for the treatment of infections caused by β-lactamase positive isolates of M. catarrhalis. Oral cephalosporins could also be an option. In case of allergy to β-lactam antibiotics tetracycline, macrolides or azithromycin could be administered. Chloramphenicol should be avoided because of its toxicity.

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Sažetak

ANTIBIOTSKA OSJETLJIVOST I PROIZVODNJA ß-LAKTAMAZE KOD IZOLATA MORAXELLA CATARRHALIS IZ ZAGREBA, HRVATSKA

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Ključne riječi: Moraxella-Branhamella catarrhalis – učinak lijekova; Proteiinfekcijski lijekovi – farmakologija; Infekcije dišnog trakta – mikrobiologija; ß-laktamae – biosinteza; Hrvatska