ERTAPENEM RESISTANCE AMONG EXTENDED-SPECTRUM-B-LACTAMASE-PRODUCING KLEBSIELLA PNEUMONIAE ISOLATES

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Abstract. Correct detection of carbapenemase-producing strains is of crucial importance. Carbapenem resistance in Klebsiella pneumoniae is rare. Aim: The purpose of our study was to analyze the resistance to carbapenems of Klebsiella pneumoniae strains isolated from different pathological products. Methods: The resistance mechanism of 58 Klebsiella pneumoniae isolates, with decreased susceptibility to carbapenems collected from 2007 to 2009 was investigated. Detection of the resistance mechanisms included a screening step, an optional phenotypic confirmatory step, followed by a genotypic step. Results: Hyper production of ESBL type enzymes combined with porin loss is regarded as the main mechanism of resistance. Analysis of β-lactamases showed that all isolates possessed ESBL genes. Other β-lactamases belonging to class A, including bla-KPC-1, class B or OXA-type carbapenemases, were not detected. Conclusions: The emergence of multiresistant Klebsiella pneumoniae isolates is of major concern and highlights the need for further surveillance.

Keywords: K. pneumoniae, carbapenemases, KPC, ESBL, Enterobacteriaceae

Introduction

Klebsiella pneumoniae is a Gram negative bacillus, member of the Enterobacteriaceae family, which produces urinary tract infections, systemic infections, pneumonia, and abdominal infections [1].

Carbapenems are considered reserve antibiotics to treat severe infections with multiresistant Gram-negative bacilli, including those with extended-spectrum beta-lactamases. Resistance to carbapenems is less prevalent in Enterobacteriaceae [2] and was first reported a decade ago.

In some geographical areas, there have been described strains producing carbapenemases class A (serine carbapenemases) especially KPC in the northeast U.S., class B (metallo-carbapenemases) for example in Greece, India, strains producing the VIM or NDM-1 enzymes and class D (OXA carbapen-

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susceptibility was tested by the Kirby-Bauer disc diffusion method using BioRad discs (Marnes-la-Coquette, France) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). With the Vitek 2 Compact system MICs were determined between two break points. Using E-test strips MICs were obtained for certain antibiotics. Strains reported as having ESBL phenotype by Vitek 2 Compact were confirmed by double disk method (ceftazidime/clavulanic acid, cefotaxime/clavulanic acid, cefepime/clavulanic acid). For all antibiotics, the testing was carried out by at least two different methods, and for some antibiotics, three different methods were used.

Investigation of resistance mechanisms to carbapenems

Phenotypic testing for mechanisms of resistance to carbapenems was made by DD synergy method by inoculating Mueller-Hinton agar plates (Oxoid UK) with a suspension of test strain adjusted to 0.5 McFarland and application of carbapenems disks ETP (ertapenem) 10μg, MEM (meropenem) 10μg and IPM (imipenem) 10μg and, at 10mm, a 300μg APB (boronic acid) disk (Bioanalise-Ankara) [4]. The expansion of the area of inhibition in the disk area between the carbapenem and the inhibitor (synergism) was considered a positive result.

Similar testing was performed for EDTA. A plate of Mueller-Hinton agar (Oxoid UK) was inoculated with a suspension of the strain adjusted to 0.5 McFarland and then we applied carbapenem disks ETP (10µg), MEM (10µg) and IPM (10µg) and, at 10mm, a filter paper disk inoculated immediately after application with 10µl 0.1 M EDTA (ethylene diamine tetra-acetic acid) (Sigma-Aldrich, Germany). Identical inhibition in the expanding area of carbapenem and disk inhibitor (synergism) was considered positive result [5].

E. coli ATCC 25922 and P. aeruginosa ATCC 27853 were used as reference strains for quality control methods.

The modified Hodge test (figure 1) was performed according to CLSI instructions [6, 7]: a 0.5 McFarland suspension of E. coli strain ATCC 25922, diluted 1/10 was inoculated on an agar plate of 90 mm. Mueller-Hinton (Oxoid UK) with a swab. After about 10 minutes, a disk of ETP (10 μg) was applied in the center of the plate. With a 10 μl loop, 3 to 5 colonies of test strain grown overnight were inoculated on the plate in the form of a straight line out from the edge of the disk, of at least 20-25mm in length. Enhanced growth around the test organism streak at the intersection of the streak and the zone of inhibition was interpreted as a positive result. K. pneumoniae strain ATCC BAA-1705 was considered a positive control and K. pneumoniae strain ATCC BAA-1706, negative control.

PCR analysis was performed to confirm the presence of the carbapenemases [8]. Strains were analyzed for bla-KPC1, bla-GES-4, bla-IMP, bla-OXA, bla-CTX, bla-TEM and bla-SHV. The method used is called direct because the colonies grown on solid medium are suspended directly in ultra-pure water and then added to the reaction mixture. PCR products were visualized in 2% agarose gel and images were captured using the Bio-Profil gel documentation system (Vilbert Lourmat, Marne-La-Vallee Cedex 1, France). Using this technique we eliminated the step of DNA isolation and laboratory and personnel contamination is minimized [9].

Optimization of reaction conditions was made after McPherson and Moller respectively Roux [10, 11].

Statistical Analysis

Data were stored and analyzed with Whonet 5.5.

Results

During January 2007 - December 2009, we isolated several strains of K. pneumoniae with reduced sensitivity or resistance to ertapenem and meropenem. From a total of four patients we isolated the same strain from different pathological products and from the remaining patients were isolated single strains. Only the first isolate per patient was included in our study. 85% of strains were isolated from urine, six strains were isolated from blood cultures, 3 from tracheal aspirates and 2 from drains. Regarding the origin, 85% of the strains were isolated from surgical departments, nine from the department of infectious diseases and
two isolates were from the intensive care unit. Most of the patients had severe disease requiring surgery and prolonged hospitalization. The age of patients ranged between 11-86 years with a median average of 56.72 years and sex distribution was as follows: 36.5% women and 63.46% men. Table I details the antibiogram results of the 58 strains.

All strains showed positive results for the ESBL test.

In terms of MIC values, all isolates had an MIC >8 mg/ml for ertapenem. For imipenem we obtained an MIC between 0.094 to 0.5 mg/ml with a geometric average of 0.193, MIC90 0.19 and MIC50 0.25. Meropenem values were: MIC between 0.38 to 6 mg/ml, geometric mean 0.702, MIC 90 0.5 and MIC50 6. For aminoglycosides, MIC values varied as follows: for amikacin between 1.5 and 256 mg/ml, for netilmicin between 3 and 256 mg/ml.

6 strains (11.53%) showed synergy between ertapenem and boronic acid, but not meropenem and imipenem with boronic acid. No strain showed synergy with ertapenem and EDTA test. 16 strains (30.76%) had positive modified Hodge test.

After PCR analysis all strains were negative for bla-KPC1, bla-GES4, bla-IMP, and bla-OXA. All isolates possessed ESBL genes: 52 (90%) strains were positive for bla-TEM, 48 (83%) strains were positive for bla-SHV and 36 (62%) strains were positive for bla-CTX-M.

### Discussion

*K. pneumoniae* is responsible for 8% of the infections occurring in hospitals. The emergence of resistance to carbapenems raises serious problems in the future therapy for these infections. Available EARSS data show that in 2008 in Greece, 36.6% of *K. pneumonia* strains were carbapenemases producing. In Israel carbapenemases producing strains have evolved as follows: 0.3% in 2005, 11% in 2006, 19.3% in 2008.

<table>
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<tr>
<th>Cod</th>
<th>Antibiotic</th>
<th>%R</th>
<th>%I</th>
<th>%S</th>
<th>%R 95%CI.</th>
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<tr>
<td>ATM</td>
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<td>0</td>
<td>0</td>
<td>90.2-100</td>
</tr>
<tr>
<td>CTX</td>
<td>Cefotaxime</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>91.4-100</td>
</tr>
<tr>
<td>FOX</td>
<td>Cefoxitin</td>
<td>84.6</td>
<td>3.8</td>
<td>11.5</td>
<td>71.3-92.7</td>
</tr>
<tr>
<td>CAZ</td>
<td>Ceftazidime</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>91.4-100</td>
</tr>
<tr>
<td>CRO</td>
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<td>0</td>
<td>0</td>
<td>90.9-100</td>
</tr>
<tr>
<td>CXM</td>
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<td>100</td>
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</tr>
<tr>
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<td>0</td>
<td>92.3</td>
<td>2.5-19.4</td>
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<tr>
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<td>Ertapenem</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>GEN</td>
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<td>0</td>
<td>5.8</td>
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<td>0</td>
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<tr>
<td>MEM</td>
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<td>96.2</td>
<td>0.0-37.1</td>
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<tr>
<td>TGC</td>
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<td>16.3</td>
<td>55.8</td>
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<tr>
<td>NET</td>
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<td>75.6</td>
<td>9.8</td>
<td>14.6</td>
<td>59.3-87.1</td>
</tr>
</tbody>
</table>

Table I. Percentages of resistance to antibiotics and 95% confidence interval
produced carbapenemases [3].

Imipenem and meropenem MIC values were low in our study. Carbapenem minimum inhibitory concentrations can exhibit considerable variation depending on the presence of different resistance mechanisms. Special attention should be paid to strains with MICs > 2 mg/ml because these strains are sensitive according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints but should still be tested for the presence of a carbapenemase gene. If the strains are tested through the disc diffusion method, values generally fit the intermediate category and sometimes the resistant category [15].

In our strains we outline a diagnostic profile: ertapenem MIC < meropenem MIC > imipenem MIC, profile seen in both UK and Italy strains and this profile suggest lack of porins OmpK35 and OmpK36 and excess production of β-lactamases CTX-M-15 together with the TEM-1 and OXA-1 [16].

A positive modified Hodge test (MHT) indicates the possible existence of a carbapenemase but cannot specify its type. Hodge test sensitivity and specificity described by Lee et al. in 2001 varies depending on the antibiotic tested. Sensitivity is 96.7% when using imipenem and specificity is 94% when using meropenem [17]. MHT frequently gives false-positive results with CTX-M ESBL, AmpC hyper-producing Enterobacteriaceae and strains that show porin modification [14, 18]. However for detection of class A carbapenemases the specificity of the modified Hodge test can be increased by modifying the test as described by Pasteran et al. [19].

The boronic acid synergy test also indicates that strains may produce a KPC-type carbapenemase. The boronic acid synergy test, described by many authors as a specific and sensitive test for carbapenemase detection, still raises issues regarding performance and interpretation [20, 21].

The inhibition mechanism is unknown in this method. There are many false positive results due to high level expression of AmpC type cephalosporinases and altering of the porins [18, 22].

A negative synergy test with EDTA excludes the possibility that our strains produce a metallo carbapenemase. We screened all 58 carbapenem-resistant K. pneumoniae isolates for the presence of bla- KPC-1and bla-IMP. Following the molecular analysis the strains were negative for both KPC and metallo carbapenemase type MBL (IMP). An important number of isolates tested positive for bla-TEM, bla-SHV and bla-CTX-M.

The limits of our study are that we could not perform the molecular analysis of several genes that confer resistance to carbapenems, nor for porines OmpK35 and 36.

As it has resulted from other studies, low levels of resistance to carbapenems can occur in the presence of a deficiency or loss of OmpK36, OmpK35 porins associated with hyper production of an enzyme type AmpC or ESBL [23-25]. Porin loss is often unstable and may impose a fitness cost, meaning that these strains rarely spread. Ertapenem is particularly affected.

Efflux may also be an increasing issue in the development of resistance to carbapenems [26].

Conclusions

Carbapenem resistance in the Enterobacteriaceae is still very uncommon but emerging.

Most K. pneumoniae strains included in our study were susceptible only to colistin, tigecycline and amikacin, so we can consider them multidrug-resistant (MDR) strains.

The most probable resistance mechanism to carbapenems in our cases is the hyper production of an ESBL combined with impermeability due to modification or porin loss. Carbapenems keep their place in the therapy of severe infections. Understanding the clinical significance of these strains is important. ESBL producers are multidrug resistant, and if carbapenems, in our case ertapenem, can be used to treat these infections, the erroneous reporting of these isolates as resistant may significantly limit the treatment options. On the other hand, if these strains are more likely to fail ertapenem treatment, the misdetection of this phenotype may have severe consequences. We intend to continue our molecular study for the presence of other carbapenemases as well as porin loss or modification. Molecular biology techniques provide a high level of specificity and are indispensable for the identification of mechanisms of resistance. Rapid detection and reporting by the laboratory of the important resistance mechanism is essential to avoid further dissemination of resistance. The microbiology laboratory should include phenotypic investigation of all multidrug resistant Enterobacteriaceae in routine practice.

References


