Molecular analysis of oxa-48 producing k. pneumoniae strains isolated from patients with catheter-associated sepsis

Ö. AKGÜL, G. BORA

Department of Pharmaceutical Microbiology, Health Sciences Institute, Faculty of Pharmacy, Van Yüzüncü Yıl University, Van, Turkey

Abstract. - OBJECTIVE: K. pneumoniae is an important cause of hospital and community-acquired infections. In particular, carbapenem-resistant strains of K. pneumoniae spread globally, increasing the public health risk. This study aims to sequence and phylogenetically analyze K. pneumoniae strains isolated from blood cultures of patients in intensive care units in our hospital.

MATERIALS AND METHODS: In this study, blood samples were collected from patients with catheter-related sepsis. Culture, biochemical, antibiotic susceptibility, and molecular tests were performed as microbiological analyses.

RESULTS: Twenty-four K. pneumoniae strains showing multidrug resistance by isolating 276 K. pneumoniae were included in the study. It was determined that they showed the highest resistance against Ampicillin, Amoxicillin/Clavulanic Acid, Ceftazidime, and Ceftriaxone. The comparison determined that K. pneumoniae isolates from different countries isolated from blood cultures had closeness and distance in OXA-48.

CONCLUSIONS: After multilocus sequence typing, all of our 24 K. pneumoniae isolates were determined to be ST11.

Key Words: Sepsis, OXA-48, Phylogenetic Analysis, MLST.

Introduction

Klebsiella pneumoniae is a significant cause of nosocomial infections, especially in intensive care units. In particular, the emergence and spread of carbapenem-resistant strains of K. pneumoniae (CRKP) pose a severe threat to global public health. Klebsiella pneumoniae is also a significant cause of community-acquired and healthcare-associated infections. Again, multidrug-resistant (MDR) K. pneumoniae is a significant cause of hospital-acquired and community-acquired infections, including bacteremia, pneumonia, urinary tract infections, and pyogenic liver abscesses. Carbapenem-resistant K. pneumoniae may exhibit a multi-antibiotic resistance profile against most beta-lactams, including carbapenems with non-beta-lactam antibiotics. Carbapenem resistance in K. pneumoniae is mainly caused by the transport of carbapenemase genes within plasmids, transposons, and integrons. The most common carbapenemases in Enterobacteriaceae are KPC (class A), VIM, IMP, NDM (class B), and OXA-48 (class D) types. OXA-48 was first described in a clinical isolate of K. pneumoniae from a hospitalized patient in Turkey in 2008 and is increasingly being reported in many countries. Among the various acquired carbapenemases found in K. pneumoniae strains, OXA-48 is one of the most common. Carbapenem-resistant K. pneumoniae strains producing OXA-48 are pretty common in Turkey, North Africa, India, and the Middle East. Moreover, OXA-48 is the most common carbapenemase in several European countries, including France, Spain, Belgium, and Malta. According to two reports from Spain and France, the prevalence of OXA-48 among carbapenemase-producing K. pneumoniae isolates was 85.2% and 87.2%, respectively. However, OXA-48-producing K. pneumoniae strains have been reported in relatively few numbers in North America and Canada.

Molecular identification methods are one of the most critical innovations offered by the developing technology in microbiology. Adding new molecular identification techniques benefits scientific studies carried out for different purposes. PFGE (Pulsed Field Gel Electrophoresis), PCR (Polymerase Chain Reaction), MLST (Multi Locus Sequence Typing), MALDI-OF (Matrix-Assisted Laser Desorption/Ionisation-Time of Flight), 16S rDNA sequence analysis methods are primarily used in the molecular analysis of bacte-
Molecular analysis of oxa-48 producing \( k. \) pneumoniae strains isolated from patients

MLST is a reliable method for identifying bacterial isolates using certain parts of the seven conserved basic genes (housekeeping genes). Pieces of approximately 450-500 base pairs (bp) in the interior of each gene are used by sequencing correctly from both strands by automatic DNA sequencing. Different sequences in the bacterial species are assigned different alleles for each conserved essential gene, and alleles at seven loci for each isolate define the allelic profile or sequence analysis (www.mlst.net 2010). Each species isolate is correctly identified by the series of 7 integers corresponding to the alleles in the seven conserved basic gene loci (www.mlst.net 2010). First, the polymerase chain reaction examines the isolates with this method. The result is obtained by multilocus sequence analysis of the obtained PCR products\(^{19}\). Many studies have reported that MLST was used on OXA-48-producing \( k. \) pneumoniae\(^{20,21}\). The first reported OXA-48-producing \( k. \) pneumonia case in Spain was in 2009\(^{22}\). Since then, multilocus STs producing OXA-48, including ST11, ST405, ST15, and ST16 spread within and between hospitals\(^{22-24}\). The most common blaOX-A-48-bearing \( k. \) pneumoniae clones isolated in Spain were ST11 and ST405\(^{25}\). They can be tracked by multilocus sequence typing (MLST) and are often found in the globally spread sequence type ST23 lineage\(^{26}\). An epidemic caused by ST11 hvKP-producing carbapenemase has recently been reported in China\(^{27}\).

Materials and Methods

**K. Pneumoniae Isolation, Identification, and Analysis of Antibiotic Susceptibility**

In 2019, carbapenem-resistant \( k. \) pneumoniae strains were collected from patients with catheter-associated sepsis in intensive care units (ICU) of Van Training and Research Hospital. For five days, blood culture bottles were followed in the Bactec/Alert 3D (Biomerieux, Hazelwood, MO, USA) device. Petri dishes were incubated at 37°C for 24-48 hours. The colony morphology of the cultures was evaluated. Biochemical tests such as catalase, oxidase, indole, \( H_2S \), and Gram stain were performed. Vitek 2 Compact (bioMerieux, Hazelwood, MO, USA) device was used to identify bacteria and evaluate the antibiogram test\(^{23}\). In the antibiotic susceptibility test of \( k. \) pneumoniae strains, Ampicillin (AM), Amoxicillin / Clavulanic Acid (AMC), Pyrarcillin (PRL), Piperacillin / Tazobactam (TPZ), Cefazolin (CZ), Cefuroxime (CXM), Cefuroxime Axetil (CXA), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ertapenem (ETP), Imipenem (IPM), Meropenem (MEM), Tobramycin (TOB), Amikacin (AK), Netilmicin (NET), Gentamicin (CN), Aztreonam (ATM), Ciprofloxacin (SPX), Levofloxacin (LEV), Tigecycline (TGC), Colistin (CT), Fosfomycin (FF), Nitrofurantoin (F), Trimethoprim / Sulfamethoxazole (SXT) (biomerieux, Hazelwood, MO, USA) were used by the European Committee on Antimicrobial Susceptibility Testing was performed using the MIC (mg/L) breakpoint table\(^{29}\). The modified Hodge test (MHT) was performed per the CLSI\(^{30}\) guideline to confirm the presence of carbapenemases\(^{31}\).

**Genomic DNA Extraction and Amplification of the bla\(_{\text{OXA-48}}\) Gene**

DNA extraction of bacteria was performed at Van Yüzüncü Yıl University, Faculty of Pharmacy, Pharmaceutical Microbiology Laboratory. Bacteria were inoculated in Tryptone Soy Agar (Acumedia, CA, USA) and incubated at 37°C for 24 hours. Then, DNAs of multidrug-resistant carbapenem-resistant \( k. \) pneumoniae strains were obtained using the EcoSpin Bacterial Genomic DNA kit (Echotech Biotechnology, Erzurum, AÜ, Turkey) protocol. DNA samples of bacteria were stored at -20°C.

The May Taq\({}\) DNA Polymerase (Bioline, Bio- 21105, Swedenoro, NJ, USA) protocol was used for the DNA amplification of bacteria. 10 µL of 5x MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl\(_2\)) as 25 µl final solution for Polymerase Chain Reaction (mPCR), 5 µL template DNA, 1 µL from each primer (20 µM), 1 µL MyTaq DNA polymerase and 8 µL PCR water (ddH2O) was calculated. PCR conditions for \( \text{bla}_{\text{OXA-48}}\) were set as 10 min at 94°C, 30 sec at 94°C, 40 sec at 52°C, 50 sec at 72°C, 5 min at 72°C, and 40 cycles. HyperLadder\({}\) marker (50 Base Pair, Bioline, Memphis, TN) evaluated amplicon sizes. Amplicon products of bacteria were run on a 1.5% agarose gel for 1 hour at 100 Volts in a Thermo EC300XL2 electrophoresis device. Amplicons were visualized using the Bio-Print-ST4 (Vilber Lourmant, Marne-la-Vallée, France) device. \( \text{bla}_{\text{OXA-48}}\) gene amplification of isolated and identified bacteria
was performed using the F: 5'-GCTGGTTAAG-GATGAACAC-3'; R: 5'-CATCAAGTTCAAC-CCAACCG -3' primer.

**bla**\textsubscript{OXA-48} Gene Sequence and Phylogenetic Analysis

Before analysis, **bla**\textsubscript{OXA-48} positive samples were purified with a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Deutschland, GmbH, Germany). PCR products with primers encoding the **bla**\textsubscript{OXA-48} gene region were packaged appropriately and sent to Sentebiolab Co. (Ankara, Turkey) for DNA sequencing. For the analysis of the nucleotide sequences, the products were edited with the help of Bioedit Software\textsuperscript{32}. The final consensus sequences of four isolates selected from each ICU were subjected to "BLAST analysis" (http://www.ncbi.nlm.nih.gov/BLAST) in the GenBank database, and the similarity rates were compared with isolates reported from different sources. Genetic distances were calculated using the Clustal W model parameter in MEGA 7.0. The **bla**\textsubscript{OXA-48} phylogenetic analysis dataset was created from the nucleotide sequences of 27 isolates. Some bacterial sequences were used as "outgroups" when constructing the phylogenetic tree. Phylogenetic analyses and tree generation were performed using the "Constructed/Test Neighbor" method with 1000 copies of bootstrap in MEGA 7.0 software\textsuperscript{33}. The phylogenetic tree includes sequences from Turkey and selected sequences from different countries.

Multilocus sequence typing (MLST), Diancourt et al.\textsuperscript{34}, and sequence types (STs) were assigned via the Institut Pasteur (Paris, France) database (http://bigdb.pasteur.fr/ klebsiella/klebsiella.html). Sequences of individual loci and merged sequences of all seven MLST loci for each isolate were analyzed for their diversity using DIVEIN\textsuperscript{35}. Clonal groups were identified based on STs sharing six loci (sin-

<table>
<thead>
<tr>
<th><strong>K. pneumoniae</strong> (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANT</strong></td>
</tr>
<tr>
<td><strong>ANT</strong></td>
</tr>
<tr>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24</td>
</tr>
<tr>
<td>AM</td>
</tr>
<tr>
<td>AMC</td>
</tr>
<tr>
<td>PRL</td>
</tr>
<tr>
<td>TPZ</td>
</tr>
<tr>
<td>CZ</td>
</tr>
<tr>
<td>CXM</td>
</tr>
<tr>
<td>CXA</td>
</tr>
<tr>
<td>FOX</td>
</tr>
<tr>
<td>CAZ</td>
</tr>
<tr>
<td>CRO</td>
</tr>
<tr>
<td>FEP</td>
</tr>
<tr>
<td>ETP</td>
</tr>
<tr>
<td>IPM</td>
</tr>
<tr>
<td>MEM</td>
</tr>
<tr>
<td>TOB</td>
</tr>
<tr>
<td>AK</td>
</tr>
<tr>
<td>NET</td>
</tr>
<tr>
<td>CN</td>
</tr>
<tr>
<td>ATM</td>
</tr>
<tr>
<td>SPX</td>
</tr>
<tr>
<td>LEV</td>
</tr>
<tr>
<td>TGC</td>
</tr>
<tr>
<td>CT</td>
</tr>
<tr>
<td>FF</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>SXT</td>
</tr>
</tbody>
</table>

"Ant: Antibiotics; R: Resistance; I: Intermediate; S: Susceptible."
Molecular analysis of oxa-48 producing k. pneumoniae strains isolated from patients

Ethics Committee Approval

The Clinical Research Ethics Committee of Van Training and Research Hospital approved the design of our study with the decision number 2018/02 on 25/01/2018.

Results

The microbiological analysis yielded 276 K. pneumoniae isolated from blood cultures. It was determined that 24 (8.6%) of these isolates were K. pneumoniae strain showing multidrug resistance and blaOXA-48 positive and were included in the study. The antibiotic susceptibility analysis of these strains determined the highest resistance against AM, AMC, CAZ, and CRO. In addition, ETP, IPM, and MEM resistance distribution ratios were the same. All K. pneumoniae isolates were sensitive to CT (Table I).

The sequence analysis results showed that the gene sequences of blaOXA-48 isolates of 24 K. pneumoniae isolates were the same. The comparison showed that K. pneumoniae isolates isolated from blood cultures of different countries had close or distant proximity within the “OXA-48 group” (Figure 1). It was observed that while the proximity was closer, especially to the European, Asian and African isolates, the genetic proximity was distant to the American isolates. It was determined that the blaOXA-48 genetic code of our bacteria could be seen in different species of Klebsiella (LC545850). The accessory numbers of our K. pneumonia isolate MW766893 (Seq1), MW766894 (Seq2), MW766895 (Seq3), and MW766896 (Seq4) were obtained from the world gene bank. After multilocus sequence typing, all of our 24 K. pneumoniae isolates were determined to be ST11.

Discussion

Klebsiella pneumoniae is a major cause of nosocomial infections, especially in intensive care units. In the last two decades, K. pneumoniae encoding antibiotic resistance genes, including carbapenem resistance, has emerged. Carbapenem resistance mediated by plasmid-encoded carbapenemases is a major public health threat as these enzymes can hydrolyze nearly all commonly used β-lactam antibiotics. The most common carbapenemases in Enterobacteriaceae are types KPC (Class A), VIM, IMP, NDM (Class B), and OXA-48 (Class D). OXA-48 was first described in a clinical isolate of K. pneumoniae from a patient hospitalized in Turkey in 2001.

Later, carbapenem-resistant K. pneumonia spread to the world. While the rate of meropenem-resis-
tant *K. pneumoniae* was 2.9% in 2005 in China, it was reported to be 26.8% in 2019. The effect of infections caused by resistant *K. pneumoniae* on mortality has also become significant. For example, the 30-day mortality rate due to circulatory system infections in Italy has been reported as 41.6%. In this study, 276 *K. pneumoniae* were isolated from blood cultures taken from patients with catheter-related bacteremia. It was determined that 24 (8.6%) isolates were *K. pneumoniae* strains that showed multidrug resistance and were bla<sub>OXA-48</sub> positive. OXA-48 positive *K. pneumoniae* isolation was performed in each intensive care unit, where blood cultures were collected. These intensive care patients were found to be in the risk group. Antimicrobial resistance has been recognized as a global public health crisis by organizations like the UN and WHO. WHO’s Global Action Plan for Antimicrobial Resistance has highlighted the need for research to fill data gaps in the incidence of infections caused by antimicrobial-resistant pathogens. It has been found that *K. pneumoniae* is resistant to many antibiotics, especially third-generation cephalosporins such as cefotaxime, ceftriaxone, and ceftazidime. An Indian study reported that strains of *K. pneumoniae* isolated from blood cultures showed significant resistance to cefotaxime, carbapenems, and piperacillin-tazobactam. In addition, it was determined that there was a significant increase in tigecycline resistance over the years. It has been reported that the resistance of carbapenem-resistant *K. pneumoniae* strains to antibiotics other than tetracycline increased significantly between 1998 and 2010. Cross-resistance has been lower than tetracycline. A Russian study reported that all *K. pneumoniae* isolates isolated from newborns with sepsis were resistant to ampicillin. In addition, all *K. pneumoniae* isolates were susceptible to meropenem, amikacin, and ciprofloxacin. Our study showed multidrug-resistant *K. pneumoniae* strains showed the highest resistance against AM, AMC, CAZ, and CRO due to antibiotic susceptibility analysis. In addition, the distribution rates of resistance to carbapenem drugs (ETP, IPM, and MEM) were the same. It was determined that all *K. pneumoniae* isolates were sensitive to CT, and this antibiotic could be highly effective in the treatment. It was concluded that susceptibility testing is essential because all *K. pneumoniae* isolates isolated from patients with catheter-related sepsis in intensive care units have different antibiotic resistance profiles.

It has been observed that ST clones may show regional differences worldwide. For example, carbapenem-resistant dominant clones ST258 and ST512 have been reported from America and Southern Europe. It has been reported that ST11 is more prevalent than ST258 or ST512 in Europe. Multiple OXA-48-producing multilocus ST clones, including ST11, ST405, ST15, and ST16, have spread within and between hospitals. A study from Spain reported that 30 of the OXA-48 positive *K. pneumoniae* strains were ST11, and the others showed different ST characteristics. A study conducted in Germany reported that 15 of 16 MDR *K. pneumoniae* strains obtained from 12 patients were bla<sub>OXA-48</sub> positive. They reported that the ST147 variant had the highest rate. In this study, the closest similarity of 24 OXA-48 positive *K. pneumoniae* strains that we isolated from intensive care units was to *K. pneumoniae* isolated from Egypt. It also showed similarity to the *K. pneumoniae* strain isolated from blood culture in Turkey. Our isolates were similar to the *K. variicola* strain isolated from the blood culture in Japan. It has been observed that our OXA-48 gene may show prevalence among different species. It has been revealed that our OXA-48 gene clones may also show differences from the OXA-48 gene clones isolated from blood. As a result of our MLST analysis, it was determined that all of the *K. pneumoniae* strains carrying the OXA-48 gene were ST11 clones. It has been determined that this clone, widely found in Europe, is also seen in our hospital. ST11 *K. pneumoniae* strains pose a severe problem in terms of public health due to their characteristics, such as being hypervirulent, multidrug-resistant, and transferable. ST11 carbapenem-resistant *K. pneumoniae* is difficult to eradicate from the circulatory system and can cause death.

**Conclusions**

As a result, it was considered critical to reveal the antibiotic resistance properties of *K. pneumoniae* strains isolated from patients with catheter-related sepsis. Gene traits and prevalence in these isolates need to be investigated in detail. Phylogenetic and sequence analysis of strains with carbapenem resistance in intensive care units were crucial. It is imperative to contribute to the surveillance system by analyzing *K. pneumoniae* strains with MLST in the fight against nosocomial infections.
Molecular analysis of oxa-48 producing K. pneumoniae strains isolated from patients

Conflict of Interest
The authors declare that they have no conflict of interests.

Ethics Approval
This retrospective study was conducted following the Ethical Principles of the Declaration of Helsinki and approved by Van Yüzüncü Yıl University Clinical Research Ethics Committee (2018/02 on 25/01/2018).

Informed Consent
With the approval of the Ethics Committee, patient consent forms were provided.

Funding
The authors received no financial support for this article’s research, authorship, and/or publication.

Authors’ Contribution
The study concept and design, data acquisition, data analysis and interpretation, and manuscript writing were prepared by Ömer AKGÜL.

ORCID ID
Ömer AKGÜL: 0000-0002-8757-2970

Data Availability
The corresponding author’s data supporting this article are available on reasonable request.

References
15) Ellis C, Chung C, Tijet N, Patel SN, Desjardins M, Melano RG, Toye B. OXA-48-like carbapenemase-producing Enterobacteriaceae in Ottawa,

17) Kiran F, Osmanapaçoğlu Ö. Laktik Asit Bakteriyle 7730 Martínez-García L, Martínez-Martínez L, Merino


Molecular analysis of oxa-48 producing *K. pneumoniae* strains isolated from patients


