



# Molecular Analysis of Ndm-1 Positive *Klebsiella pneumoniae* Strains Isolated From Patients With Bacteremia Due to Urinary System Infection

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## Abstract

**Introduction:** Health concerns about *Klebsiella pneumoniae*, which causes urinary tract infections (UTIs) and sepsis, are increasing worldwide. In addition, New Delhi metallo- $\beta$ -lactamase (NDM) carrier *K. pneumoniae* and other Gram-negative bacteria appear to cause serious clinical problems. This study aimed to reveal NDM-1 positive *K. pneumoniae* carrier status, molecular properties, and antibiotic resistance differences of patients who have bacteremia due to urinary system infections.

**Methods:** Blood culture, biochemical tests, Vitek2, polymerase chain reaction, sequencing, phylogenetic analysis, and multilocus sequence typing (MLST) methods were used for the microbiological analysis of *K. pneumoniae* strains.

**Results:** Overall, 146 *K. pneumoniae* strains were obtained from the cultures, 16 of which were found to be NDM-1 positive. Although these strains were discovered to have resistance to the authorized antibiotics, they were sensitive to gentamicin (CN), colistin (CT), and trimethoprim/sulfamethoxazole (SXT). Furthermore, it was found that the resistance rates of carbapenem (ertapenem (Etp), imipenem (Ipm), and meropenem (Mem)) were high. All *K. pneumoniae* strains represented extended-spectrum beta-lactam resistance. It was found that the phylogenetic affinities of *K. pneumoniae* strains were higher with Asian strains. Five and eleven *K. pneumoniae* isolates were determined to be ST17 and ST147 type variations, respectively, as a conclusion of the MLST study.

**Conclusions:** It was observed that the presence of NDM-1-positive *K. pneumoniae* may pose a serious problem in patients with bacteremia caused by UTI. It has been demonstrated that it is important to develop preventive and control measures in the hospital by considering NDM-1-positive *K. pneumoniae* strains with multi-drug resistance.

**Keywords:** *Klebsiella pneumoniae*, NDM-1, Blood, Sequence, Phylogenetic analysis

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## Introduction

*Klebsiella pneumoniae* was first isolated and obtained from the lung tissues of pneumonia-associated patients by Carl Friedlander (1). *K. pneumoniae* bacteria play an important role in hospital infections (2). Several studies have determined that generally 18-31% of all hospital infections and 3%-17% of community-acquired infections are caused by *K. pneumoniae* bacteria. Several studies have also reported that mortality due to *K. pneumoniae* has been responsible for mortality incidence rates of 30% and 54% in intensive care units (3,4). Moreover, it has been observed that approximately 33% of Gram-negative infections which lead to pneumonia, urinary system infections (cystitis), endocarditis, surgical-wound infections, and septicemia are caused by these bacteria (5). In addition, this organism plays a role in important infections, including severe infections such as pyogenic liver abscesses, necrotizing pneumonia, and endogenous endophthalmitis (6). Many researchers concur that these severe infections generally occur in patients who have

been hospitalized, in immunocompromised individuals, as well as in patients who are on a routine basis being cured with  $\beta$ -lactams and distinct antibiotics which are counteractive Enterobacteriaceae (7). Urinary tract infection (UTI) is defined as any infection that may occur in the urinary tract. About 40% of women are affected by bacterial infections in their life. UTIs can induce the potentially fatal condition sepsis, but most infections are not as serious (8). It has been determined that severe sepsis in North America includes more than 500 000 people per year with a mortality rate of approximately 40%, depending on several factors and most importantly the severity of sepsis (9,10). These types of infections are mainly caused by bacteria (e.g., *K. pneumoniae*), though other microorganisms such as viruses, fungi, and protozoa can also lead to sepsis (11,12).

Antimicrobial resistance of Enterobacteriaceae is associated with significant problems such as prolonged hospitalization, high healthcare cost, and high morbidity and mortality (13). Since Enterobacteriaceae frequently



develop resistance to fluoroquinolones and extended-spectrum beta-lactamase (ESBL), carbapenems have emerged as the antibiotic to be selected as the primacy medicine for treating Gram-negative pathogens, which are resistant to multiple drugs. However, the increased utilization of carbapenems has resulted in putting forth several carbapenem-resistant Enterobacteriaceae (CRE) strains (14,15). In the midst of Enterobacteriaceae, clinically meaningful carbapenemases are class A (KPC), class B (Verona integron-encoded metallo- $\beta$ -lactamase [MBL], imipenemase, and New Delhi metallo- $\beta$ -lactamase [NDM]), and in class D (OXA-48) groups. They are almost always present in *K. pneumoniae* strains and are frequently dangerous in connection with outbreaks or hospital infections (16). Carbapenem resistance is principally resulting from the production of carbapenems, which are  $\beta$ -lactamases that can hydrolyze all  $\beta$ -lactam antibiotic groups, including carbapenems, which are considered the most powerful class (17). NDM is the first bacterial enzyme identified in *K. pneumoniae* and *Escherichia coli* in 2008 as it was isolated from a Swedish patient undergoing treatment in India (18). Since then, evidence of both chromosomal integration and plasmid carriage in a variety of Gram-negative organisms has demonstrated the mechanism causing this resistance (19). Thereafter, Enterobacteriaceae with NDM-1 have been shown to widely spread in Pakistan, India, Bangladesh, and the UK (20,21). Since then, reports of Enterobacteriaceae with NDM-1 have been made all over the world (22,23). Patients under medical treatment attention previously in India traveling to the United States and Canada were the first NDM-1-producing Enterobacteriaceae instances found in 2010 (24,25). Initially, the strains of NDM-1-producing isolates in the Middle East and Arabian Peninsula were determined by Poirel et al (26). In Lebanon and Kuwait, several similar cases of Enterobacteriaceae carrying NDM-1 have been found as well (27,28). Recently discovered carbapenem-resistant enterobacterial isolates from Oman had 11 of them as NDM-1 positive, 5 of them as OXA-48 positive, and 1 of them as NDM-1 plus OXA-181 positive, suggesting the advent of carbapenems producers in the geographical area (29).

The objective of the current research is to reveal the frequency of the occurrence of the NDM-1 carriage of *K. pneumoniae* isolates obtained from patients, who are diagnosed with UTIs and sepsis. In addition, it was also aimed to analyze the existence of the risk posed by multiple antibiotic-resistant strains on public health. Furthermore, it was aimed to evaluate the similarities of the strains by performing sequence and phylogenetic analysis of NDM-1 positive *K. pneumoniae* isolates.

## Materials and Methods

### *The Isolation, Identification, and Analysis of the*

### *Susceptibility of the K. pneumoniae Strain to Antibiotics*

The intensive care units (ICUs) at our hospital collected carbapenem-resistant *K. pneumoniae* strains from bacteremia patients diagnosed with UTIs in 2019. Five days were spent monitoring blood culture bottles using the Bactec/Alert 3D (bioMérieux, USA) system. Inoculations from blood culture bottles were performed on 5% sheep blood agar (Acumedia, USA), MacConkey Agar (Oxoid, UK), and Eosin Methylene Blue (EMB, Oxoid, UK) agar. For 24-48 hours, Petri plates were incubated at 37 °C. The colony morphologies of cultures underwent assessment. Gram-staining and biochemical assays such as catalase, oxidase, indole, and H<sub>2</sub>S were conducted. The antibiogram test was evaluated and bacteria were identified using the Vitek 2 Compact (bioMérieux, USA) instrument (30). Different antibiotics were used for the antibiotic susceptibility testing of the *K. pneumoniae* strains, including ampicillin (AM), amoxicillin/clavulanic acid (AMC), amikacin (AK), piperacillin (PRL), piperacillin/tazobactam (TPZ), cefepime (FEP), cefazolin (CZ), and ceftazidime (FOX). The other antibiotics were cefuroxime (CXM), ciprofloxacin (SPX), cefuroxime axetil (CXA), Colistin (CT), ceftazidime (CAZ), ceftriaxone (CRO), ertapenem (ETP), fosfomycin (FF), imipenem (IPM), gentamicin (CN), levofloxacin (LEV), meropenem (MEM), and nitrofurantoin (F). In addition, the remaining applied antibiotics included netilmicin (NET), aztreonam (ATM), tobramycin (TOB), tigecycline (TGC), and finally trimethoprim/sulfamethoxazole (SXT). The testing was conducted with the guidelines of the European Committee on Microbiological Resistance by utilizing the minimal inhibitory concentration (mg/L) cut-off value table (31). According to the Clinical and Laboratory Standards Institute, 2012 guideline, the modified Hodge test was performed to determine the presence of carbapenemases (32,33).

### *Genomic DNA Extraction and Amplification of the blaNDM-1 Gene*

DNA was extracted from bacteria in the Laboratory for Pharmaceutical Microbiology at Van Yüzüncü Yıl University. On Tryptone Soy Agar from Accumedia in the United States, bacteria were grown and then incubated for 24 hours at 37 °C. Then, DNAs belonging to the multi-drug resistant carbapenem-resistant *K. pneumoniae* strains were recovered using the EcoSpin Bacterial Genomic DNA kit (Echotech Biotechnology, Turkey) methodology. Bacterial DNA samples were kept at -20 °C.

The MyTaq™ DNA Polymerase (Bioline, Bio-21105) protocol was utilized for the DNA amplification of bacteria. For polymerase chain reaction (mPCR), 10  $\mu$ L of 5x MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl<sub>2</sub>), 5  $\mu$ L of template DNA, 1  $\mu$ L of each primer (20  $\mu$ M), 1  $\mu$ L

of MyTaq DNA polymerase, and 8 µL of nuclease-free water were calculated as 25 µL of the final solution. PCR conditions for NDM-1 were set as 40 cycles at 94 °C for 10 minutes, 94 °C for 30 seconds, 52 °C for 40 seconds, 72 °C for 50 seconds, and 72 °C for 5 minutes. HyperLadder™ marker (50 Base Pair, Bioline, USA) was used to evaluate amplicon sizes. Amplicon products of bacteria were run on a 1.5% agarose gel for an hour at 100 V with a Thermo EC300XL2 electrophoresis device. Amplicons were visualized using the Bio-Print-ST4 (Vilber Lourmat, France) device. The *NDM-1* gene amplification of isolated and identified bacteria was performed using the following primer: F: 5'-GGTTTGGCGATCTGGTTTTTC-3', R: 5'-CGGAATGGCTCATCACGATC-3'.

### **NDM-1 Gene Sequence and Phylogenetic Analysis**

Prior to the analysis of the results, NDM-1 positive samples were purified using a commercial purification kit (High Pure PCR Cleanup Micro Kit, Roche, Germany). The *NDM-1* gene region's primers and PCR products were carefully packaged and submitted to the Sentebiolab firm in Ankara for DNA sequencing. BioEdit software was used to edit the products prior to the analysis of nucleotide sequences (34). The "BLAST analysis" (<http://www.ncbi.nlm.nih.gov/BLAST>) was performed on the final consensus sequences of a total of four isolates chosen from each ICU, and similarity rates were compared with isolates published from various sources. When using MEGA 7.0, the Clustal W model parameter was utilized to determine genetic distances. The nucleotide sequences of a total of 27 isolates were employed to construct the blaNDM-1 phylogenetic analysis dataset. The "outer groups" of some bacterial sequences were applied to build the phylogenetic tree. With 1000 bootstrap replications, phylogenetic analyses and tree building were performed in MEGA 7.0 using the "maximum likelihood technique" method (35). Sequences from Turkey and a few other nations are included in the phylogenetic tree.

Sequence types (STs) were allocated using the Institut Pasteur (Paris, France) database (<http://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>), and multi-locus sequence typing (MLST) was conducted in accordance with the approach of Diancourt et al (36). DIVEIN was used to assess the diversity of each isolate's seven MLST loci's individual loci sequences and concatenated sequences (36). eBURST was employed to identify clonal clusters based on STs sharing six loci (single locus variations) (37). eBURST was utilized to create a population snapshot for the clonal connection of these STs with those in the Pasteur Institute database (38).

### **Results**

In general, 146 *K. pneumoniae* were isolated from blood cultures via detailed microbiological analysis. Hence, 16 (11%) of these isolates were found to be multidrug-

resistant *K. pneumoniae* strains and were NDM-1 positive, thus they were added to the study. These isolates were subjected to an examination of their antibiotic susceptibility, and it was discovered that they were susceptible to CN, CT, and SXT while being completely resistant to all other antibiotics. Furthermore, in this analysis, the ETP, IPM, and MEM resistance distribution ratios were found to be equal. All strains of *K. pneumoniae* were also observed to have an extended-spectrum beta-lactam resistance (Table 1).

Our isolates in the tree formed as a result of the phylogenetic analysis showed genetic similarity to most of the isolated NDM-1 positive *K. pneumoniae* strains that were obtained from blood, as well as other clinical organic materials. It was determined that there was a high level of affinity with NDM-1-positive *K. pneumoniae* strains isolated from sputum in China and blood in Pakistan. However, it was revealed that there was a complete difference between NDM-1-positive *K. pneumoniae* strains isolated from blood specimens in India and urine specimens in Iran. Accessory numbers of NDM-1 positive *K. pneumoniae* isolates which were isolated from the blood of the sick who were being treated in ICUs were MW750220, MW750221, MW750222, and MW750223, respectively (Figure 1). Based on the MLST analysis, it was found that 5 and 11 of our NDM-1 *K. pneumoniae* isolates were ST17 and ST147 variants.

### **Discussion**

The most prevalent strain of carbapenem-resistant Enterobacteriaceae (CRE), *K. pneumoniae*, developed as a result of the need to treat ESBL infections with carbapenem-containing antibiotics. In 2013, the Centers for Disease Control and Prevention declared CRE to be an urgent public health issue requiring rapid attention in the United States. It has been observed that *Klebsiella* has been the cause of approximately 80% of 9,000 infections due to CRE (39). The accessory genome can control the occurrence of carbapenem resistance, sometimes caused by changes in the core genome itself. Furthermore, Carbapenem resistance in *K. pneumoniae* may occur as a result of the overregulation of efflux pumps (40), alteration of outer membrane pores in the nuclear genome (41), and overproduction of ESBL or AmpC β-lactamase enzymes in the accessory genome (42). Moreover, plasmid-mediated carbapenemases are the most fighter form of carbapenem resistance. The accessory genome of *K. pneumoniae* also contained other carbapenemase species. NDM-1 in the class B group is encoded on plasmids (MBL). MBLs are distinguished by the need for zinc at their active sites, and infections with isolates that produce MBLs are constantly linked to hospitalization and travel to endemic areas (43). NDM-1, for instance, was found in a *K. pneumoniae* isolation from a Swedish patient, who had visited India (18). Since that

**Table 1.** The Analysis of the Isolates of Multidrug-resistant *K. pneumoniae* for Antibiotic Susceptibility

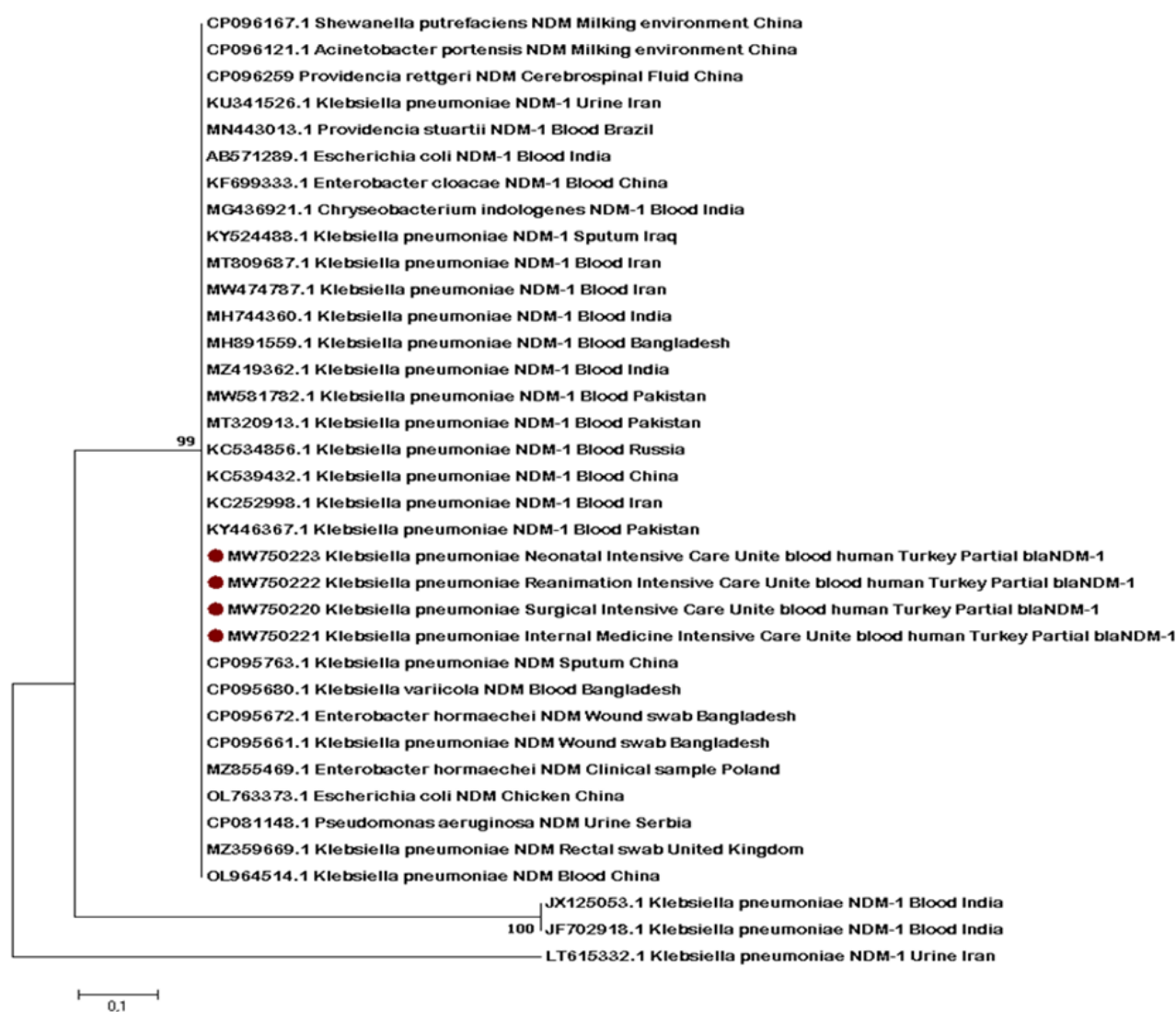
ANT	<i>K. pneumoniae</i> (N=16)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
AM	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
AMC	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
PRL	R	R	R	R	R	I	I	I	R	I	I	R	R	R	R	R
TPZ	R	R	R	R	R	I	I	I	R	I	I	R	R	R	R	R
CZ	R	R	R	R	R	R	R	R	I	I	I	R	R	R	R	R
CXM	R	R	R	I	R	I	R	R	R	R	R	I	R	R	I	R
CXA	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
FOX	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R
CAZ	R	R	R	I	R	R	I	R	R	R	R	R	I	R	R	R
CRO	R	R	R	R	I	R	R	R	R	R	R	R	R	R	R	R
FEP	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
ETP	R	R	R	I	R	I	R	R	R	R	I	R	R	I	R	R
IPM	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
MEM	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TOB	R	R	R	R	I	R	R	I	R	R	R	R	I	R	R	R
AK	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
NET	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CN	I	S	S	I	S	S	S	S	I	S	S	I	I	S	S	I
ATM	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
SPX	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
LEV	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
TGC	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
CT	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
FF	R	R	R	R	R	R	R	R	R	R	I	I	I	R	R	R
F	R	R	R	R	R	R	R	R	R	R	I	I	I	R	R	R
SXT	S	S	R	S	S	I	I	I	S	S	S	S	I	I	S	S

Note. *K. pneumoniae*: *Klebsiella pneumoniae*; R: Resistance; I: Intermediate; S: Susceptible; ANT, Antibiotics.

time, visitors to or residents of the Indian subcontinent have been closely linked to the acquisition of NDM-1 isolates (20,44). There is epidemiological evidence demonstrating that traveling to India and surrounding countries in the subcontinent is an essential risk factor for the transmission of NDM-1-producing bacteria (20,21). NDM-1-carrying multidrug-resistant bacteria have also been reported in the United Kingdom, the Balkans, and Middle Eastern nations (20,22,45). There have been reports of infections linked to NDM-1-positive strains everywhere, including India, the UK, the US, Canada, Australia, France, the Netherlands, China, Pakistan, Italy, Spain, and Canada (46). Overall, 146 *K. pneumoniae* was isolated from patients with UTIs for the current study, 16 of which were found to be NDM-1-positive. In our hospital, it was observed that the bacterial flora carrying NDM-1 poses a risk to patients and healthcare personnel.

There have been several incidences of outbreaks in Southern Asia, Europe, and the USA due to Enterobacteriaceae which produces NDM-1 (47,48).

According to the literature and records, in Canada, there have been no outbreaks caused due to NDM-1-producing organisms. Previous research has only looked at reports of one single instance of Enterobacteriaceae producing NDM-1 (25,49). The *NDM-1* gene has primarily been found in the isolates of *K. pneumoniae* and *Escherichia coli* that are not related clonally (20,50). According to a recent study, ST14, ST11, ST149, ST231, and ST147 were the most frequently discovered STs in the clinical isolates of *K. pneumoniae* producing NDM-1 from India, the United Kingdom, and Sweden (51). Additionally, it has been noted that ST14 and ST147 have been discovered in various nations (26). Additionally, the isolates of ST231 from the UK have been found as well (52,53). In addition, in India and Mauritius, ST147 has been identified among the NDM-1-producing isolates originating from Switzerland and Iraq (54,55). *K. pneumoniae* producing an NDM-1 of ST15 has also been reported from Norway (56). The only *K. pneumoniae* isolate that co-produces NDM-1 and OXA-181 belongs to ST11, which was



**Figure 1.** Phylogenetic Analysis of NDM-1 Positive *K. pneumoniae* Strains Found in Patients With Bacteremia With Urinary System Infection by “Maximum Likelihood Method”. Note. NDM: New Delhi Metallo- $\beta$ -lactamase

previously identified in India (57). In a study conducted in China, three of the *K. pneumoniae* isolates from blood were identified as ST17. None of the STs belong to the most common *K. pneumoniae* STs (ST14 and ST11) reported to have NDM-1 (51). Additionally, only one NDM-1-positive ST17 case has been confirmed in Guatemala (23). In this study, it was demonstrated that our *K. pneumoniae* isolates, which were isolated from our hospital, were ST17 and ST147 variants. It was revealed that these variants can be found in different parts of the world, as in the above-mentioned studies, as well as in our region. In addition, our isolates represented higher similarity with Asia-originated strains as a result of the phylogenetic analysis. Further, there could be genetic differences with NDM-1-positive *K. pneumoniae* strains isolated from different clinical materials in the Asian continent.

Based on the antimicrobial susceptibility test, bacteria that were resistant to NMD-1 showed substantial

resistance to the majority of antibiotics (except amikacin), having an overall resistance rate of over 50%. It has been observed that NDM-1 has a high level of resistance against drugs such as aminoglycosides,  $\beta$ -lactam antibiotics/ $\beta$ -lactamase inhibitors, and carbapenems. There is approximately up to a 100% resistance of NDM-1 against drugs such as ampicillin, doripenem, ampicillin, furadantin, cefotaxime, ceftazidime, cefuroxime, ceftriaxone sodium, and cefoxitin. Some rates of drug resistance against some carbapenems are 97.30% (meropenem), 97.30% (imipenem), and 92.00% (ertapenem). Nonetheless, it is interesting to note that NDM-1 resistance rates against drugs comprised of polymyxin B (37.93%), amikacin (32.65%), tigecycline (7.69%), and polymyxin E (2.33%) are significantly lower as compared to the other antibiotics. It was also found that NDM-1-resistant bacteria can frequently be resistant to multiple drugs, and plasmids containing blaNDM-1 can transfer the multidrug resistance gene

(58). In a study by Shibl et al (16), nine *K. pneumoniae* isolates were isolated from blood, 2 of which were found to be NDM-1-positive. It was determined that the isolates were resistant to imipenem and meropenem. It was revealed that amikacin, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole represented the highest resistance. In their study, Pons et al (59) reported that 30 NDM-1 positive *K. pneumoniae* were isolated from blood cultures. All isolates were reported to have resistance against imipenem and meropenem. In addition, they were resistant to third-generation cephalosporins, piperacillin-tazobactam, amoxicillin-clavulanic acid, monobactam aztreonam, trimethoprim-sulfamethoxazole, gentamicin, and ciprofloxacin. It was demonstrated that antibiotic susceptibilities were observed only for colistin, tigecycline, levofloxacin, and amikacin; therefore, all isolates were classified as MDR. In this study, it was found that NDM-1-positive *K. pneumoniae* strains were resistant to all antibiotics but were sensitive to CN, CT, and SXT. Furthermore, it was revealed that the distribution ratios of carbapenem (ETP, IPM, and MEM) resistance were found to be equal. All *K. pneumoniae* strains showed extended-spectrum beta-lactam resistance. Demonstrating the multi-drug resistant bacterial profile in our hospital was found to be important in terms of creating treatment protocols and contributing to surveillance programs. This way the medical community can be prepared for future eventualities and infections caused by these similar bacteria and organisms.

### Conclusion

It was determined that patients were at high risk for NDM-1-positive *K. pneumoniae* isolates that demonstrate multidrug resistance in our regional hospital. It was concluded that it is important for the hospital infection control committee to develop protection and control measures against NDM-1-positive *K. pneumoniae* in the struggle against multi-drug resistant bacteria. It was recommended that the preparation of the treatment protocols of the patients according to their antibiotic resistance status is important for a successful prognosis. Hence, this treatment protocol should become more widespread in the future for getting better results from similar situations.

### Authors' Contribution

**Conceptualization:** Ömer Akgül, Gülhan Bora.

**Data curation:** Ömer Akgül, Gülhan Bora.

**Formal analysis:** Ömer Akgül

**Funding acquisition:** Ömer Akgül.

**Investigation:** Ömer Akgül.

**Methodology:** Ömer Akgül.

**Project administration:** Gülhan Bora.

**Resources:** Ömer Akgül.

**Software:** Ömer Akgül.

**Supervision:** Ömer Akgül, Gülhan Bora.

**Validation:** Gülhan Bora.

**Visualization:** Ömer Akgül, Gülhan Bora.

**Writing-original draft:** Ömer Akgül

**Writing-review & editing:** Ömer Akgül, Gülhan Bora.

### Competing Interests

There is no conflict of interests as per the author's declaration.

### Ethical Approval

The current retrospective research was conducted in line with the ethics principles of the Declaration of Helsinki and approved by the Ethics Committee for Clinical Research of Van Yuzuncu Yıl University (20 November 2019, No.: 2019/08). The Clinical Research Ethics Committee at Van Training and Research Hospital gave its clearance to our study (Decision numbered 2018/02; dated 25/01/2018).

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### Informed Consent

Subsequent to ethics committee approval, the consent forms were filled and signed by the participating patients.

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