

## ORIGINAL ARTICLE



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## Correlation of blaNDM to Antibiotic Resistance Patterns in *Klebsiella pneumoniae* Isolates from Tertiary Care Hospital in Lahore, Pakistan

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### Abstract

This study was conducted to evaluate the antibiotic susceptibility of *Klebsiella pneumoniae* isolated from different clinical samples collected at a tertiary care hospital in Lahore, Pakistan. A total of 50 isolates were included in this study and 28 different antibiotics were used to determine the antibiotic susceptibility of isolates. The pure colonies of *K. pneumoniae* were isolated by the conventional method of bacterial identification and then the pure bacterial growths were tested for antibiotic susceptibility against different antibiotics. PCR amplification was performed to identify three virulent genes; *Klebsiella pneumoniae* Carbapenamase (KPC), Metallo-β-lactamases (NDM), IMiPenamase (IMP) followed by sequencing analysis. The *K. pneumoniae* isolates were found highly resistant to Ampicillin by 56% and Amoxicillin, Cefepime, Cefixime, Cefaclor, and Cefuroxime by 54%. The isolates were highly sensitive to Colistin (100%), Amikacin (14%), Imipenem and Meropenem (18%). The prevalence of antibiotic resistance was found more in female patients as compared to males. The prevalence of NDM gene was 22% while we were unable to detect KPC and IMP in these isolates. In the present study a prominent correlation was found between the presence of NDM gene and antibiotic resistance as this gene was found positive only in MDR strains. The co-existence of multiple drug resistance and the NDM gene is an alarming situation and demand more research to determine possible mechanisms of co-existence between blaNDM and antibiotic resistance.

**Keywords:** *K. pneumoniae*, emergence of NDM, KPC, IMP

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### INTRODUCTION

Enterobacteriaceae possesses a clinically significant mechanism of antibiotic resistance among Gram-negative bacteria. Apart being colonizers of the gut microbiota of both animals and humans found in water and soil, some of the members also serve as human pathogens. Infections range from urinary tract infections, gastrointestinal infections, pneumoniae and severe bloodstream infections (1)

One of the most common pathogens in the Enterobacteriaceae family is *Klebsiella pneumoniae*. It is Gram-negative, non-motile, encapsulated, lactose-fermenting, non-spore forming, facultatively

anaerobic, rod-shaped bacterium. *K. pneumoniae* can survive in multiple habitats because of its metabolic versatility. Besides colonizing mucosal surfaces of living body, this organism was also discovered in the soil, freshwater, root surfaces of plants and digestive tract of insects (2). It has been demonstrated to increase crop yields in agriculture conditions. As pathogenic microbe, *K. pneumoniae* has exhibited a maximum rate of extended-spectrum β-lactamases (ESBL) associated resistance to virtually all beta-lactam antibiotics except carbapenems (2).

β-lactam antibiotics are characterized by the beta-lactam ring in their molecular structure and this ring

controls their mode of action. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is one of the carbapenem-resistant *Enterobacteriaceae* (CRE), an emerging cause of antibiotic-resistant, healthcare-associated infections. CRE was listed as one of the most urgent antibiotic resistance threats by the CDC and WHO (3). Each specified ring of beta-lactam antibiotics binds to a particular penicillin-binding-protein present on the surface of bacterial cell wall and inhibits its synthesis. When colistin is combined with meropenem or rifampin the mechanism of synergistic activity is thought to be the perturbation of the Octamethyl Pyrophosphoramidate by colistin that favors the easy penetration of meropenem/rifampin at intracellular concentrations that enable inhibition of protein synthesis eventually leading to cell death (4). Previous studies also showed that colistin-rifampicin and colistin-meropenem/ imipenem could exert synergistic effects (5). However, the most important mechanism of resistance is through antibiotic degrading enzymes called ESBLs. ESBLs are a group of diverse mostly plasmid-mediated enzymes that can be produced by all *Enterobacteriaceae*. The most common and clinically most important species are *E. coli* and *K. pneumoniae*. Through hydrolysis the enzymes cause an opening of the  $\beta$ -lactam ring of penicillin, cephalosporins, monobactams, which in turn leads to an inactive form of the  $\beta$ -lactam antibiotic (2).

Co-resistance to further significant antibiotic classes such as fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides is common among ESBL-producing *Enterobacteriaceae* (EPE) and making the antibiotic treatment even more challenging. These infections are a worldwide problem, with recent reports indicating that CRE are widespread in the United States Europe, and China (6). The major worldwide threat is carbapenemase-producing *Enterobacteriaceae* (CPE) which is found resistant to carbapenemase. The most common carbapenemases were encountered within the *Enterobacteriaceae* family in the USA, Israel, and Greece where an endemic situation has been created (7,8,9). High-resolution genomic analysis of multidrug-resistant hospital outbreaks of *K. pneumoniae* through whole-genome sequencing revealed the emergence of capsules witting NDM-1 bearing *K. pneumoniae* ST15 strain, suggesting that further studies should concentrate on the diversity and spread of this specific clone (10). ST15 harboring blaNDM1 has often been reported in many countries, including Spain Croatia (11). The presence and distribution of high-risk CCRE clones, epidemic clades and successful plasmids carrying carbapenemase genes in European hospitals and their

potential cross-border spread (12). Genetic investigation of the blaKPC genes indicates that their mobility is mainly related to the extent of strain plasmid and transposon. Plasmids can carry multiple genes including multiple  $\beta$ -lactamases aminoglycosides modifying enzymes resistance genes for fluoroquinolones and trimethoprim-sulfamethoxazole virulence genes (7).

The present study was conducted to evaluate the antibiotic susceptibility of *K. pneumoniae* and to identify three virulent genes i.e., KPC, NDM, and IMP among drug-resistant *K. pneumoniae* isolate collected from a tertiary care hospital in Lahore, Pakistan.

## MATERIALS AND METHODS

### Sample Collection and Isolation of *K. pneumoniae*

A total of 410 samples were collected from patients admitted in different wards in Shalamar Hospital Institute of Health Sciences, Lahore in the duration of April 2018 to July 2018. From these 410 samples, 50 isolates of *Klebsiella pneumoniae* were isolated which were proceeded further for phenotypic and genotypic identification. These samples were inoculated on MacConkey agar. After 18 hours of incubation at  $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$  under aerobic conditions, selected colonies were identified by their colonial morphology, Gram staining and biochemical reactions (Simon citrate test).

### Antibiotic Susceptibility Testing

After isolation and identification of *K. pneumoniae*, the antibiotic susceptibility was performed by disk diffusion method according to the protocol given by CLSI guidelines (2017)(13). To standardize the inoculum density for susceptibility tests McFarland standards were used.

Commercially available antibiotic disks (OXOID) were used to determine antibiotic sensitivity testing. The following antibiotics were placed on inoculated plates; Amoxicillin (AMX), Ampicillin (AMP), Cefepime (FEP), Cefotaxime (CTX), Cefixime (CFM), Ceftazidime (CZA), Cefaclor (CEC), Cefuroxime (CXM), Imipenem (IPM), Meropenem (MEM), Amikacin (AMK), Gentamicin (GEN), Tobramycin (TOB), Doxycycline (DOX), Nalidixic Acid (NAL), Ciprofloxacin (CIP), Levofloxacin (LVX), Norfloxacin (NOR), Ofloxacin (OFX), Sulfamethoxazole-Trimethoprim (SXT), Fosfomycin (FOS), Ticarcillin (TIC), Colistin (CST), Tigecycline (TGC), Trimethoprim/sulfamethoxazole (TMP/SMX), Moxifloxacin (MXF) and Tazobactam (TZB). Five disks at 24mm from the center to center

were placed on a 90mm plate. After 48 hours of incubation period, each Petri plate was checked for a confluent lawn of growth with the clear zones of inhibition. The diameter of the zone of inhibition was measured including the diameter of disk. The zone was measured in millimeters with the help of a ruler on the backside of the plate.

#### Molecular Identification of Genes

The extraction of bacterial DNA was done using DNA extraction kit. (Wiz Prep TM) NDM, KPC and IMP genes were amplified using primer provided in Table 1. Specific bands were excised and eluted using gel elution kit (Pro mega Technologies, USA) according to the protocol given with the kit. Sequencing was performed for gene confirmation using commercial sequence services of Macrogen Inc., South Korea.

per diameter of zones (mm) for *K. pneumoniae*. The results were presented in Table 2.

#### Molecular Identification of NDM, KPC and IMP Genes

In this study, we found 22% prevalence of NDM gene. KPC and IMP gene could not be detected from any isolate (Fig. 1). The Relationship between the presences of the virulent genes of *K.pneumoniae* and gender is shown in Table 3.

#### DISCUSSION

The problem of bacterial antibiotic resistance emerged as soon as the first antibiotic became available for clinical use. All the isolates were tested for antibacterial susceptibility (15). The morbidity and mortality rates related to antibiotic-resistant *K. pneumoniae* had been recorded moderately high during this decade.

**Table No. 1: Primer Sequences of NDM, KPC and IMP Genes.**

Gene	Primer Sequence	Product Size (bp)	Annealing Temperature (°C) (1 min)	References
<i>BlaNDM</i>	F: GGTGGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	621	61	(14)
<i>BlaKPC</i>	F: CGTCTAGTTCTGCTGTCTTG R: CTGTGCATCCTTGTTAGGCG	798	63	(14)
<i>BlaIMP</i>	F: GGAATAGAGTGGCTTAAAYTCTC R: GGTAAAYAAAACAACCACC	232	63	(14)

**RESULTS** *K. pneumoniae* samples were collected from urine, blood, sputum, vaginal swab, pus, tracheal aspirate, wound swab, and folly's tip. These samples were processed for the isolation, identification and antibiotic resistance profiling and molecular characterization for NDM, KPC and IMP genes.

#### Identification of Isolates

Based on the colonial morphology, gram staining and biochemical tests, 52 isolates of *K. pneumoniae* were selected.

#### Antibiotic Susceptibility Assay

*K. pneumoniae* was screened for antibiotic sensitivity described by CLSI guideline 2017. After the application of disks and incubation at 37°C for 48hrs, isolates were marked as Resistant or Sensitive

Multiple studies have checked the relationship between the infections caused by carbapenem-resistant *K. pneumoniae* over the duration of disease (16,17,18,19,20). In the present study, out of 50 isolates, 28 were found resistant to Ampicillin and 27 to Amoxicillin, Cefepime, Cefixime, Cefaclor, and Cefuroxime. High sensitivity for *K. pneumoniae* was also found with different antibiotics of which 41 isolates were sensitive to Meropenem and 43 to Amikacin. Colistin being a 'Drug of Last Resort' (DoLR) killed each of the 50 isolates. In studies conducted in Bangladesh, it was concluded that nearly 81% Klebsiella isolates exhibited resistance to Amoxicillin and 58% isolates to Nalidixic acid (15). A previous study from Al-Najaf showed that 97% of isolates were resistant to AMC (21). A study from Iran showed that the percentage of resistance to different antibiotics were in the following order: Aztreonam (79.7%), Cefixime (67.4%), Cefpodoxime (66.2%), Cefotaxime (65.1%) and Ceftazidime (61.7%) (22). This spread of

resistance is related to the constant and indiscriminate use of antibiotics by masses without prescription but also by physician's false prescriptions. Also, wrong dosage and duration of use of antibiotics is also a reason why microbes are gradually getting resistant to antibiotics. In contrast to males, females have a higher percentage of beta-lactamase-producing *Klebsiella* infections. In a previous study of female patients the age group at 31-40 years, manifested the highest percentage (29%) (15). In males, the age groups between 50+ and 70+ showed high frequency of such infection (23). On the other hand, high frequency in females was in age groups of 70+ and 20-30+ reported men and women of elderly group were found to be very much prone to UTI (24). In a previous study from Pakistan, both males and females in the age group of 50+ were found more

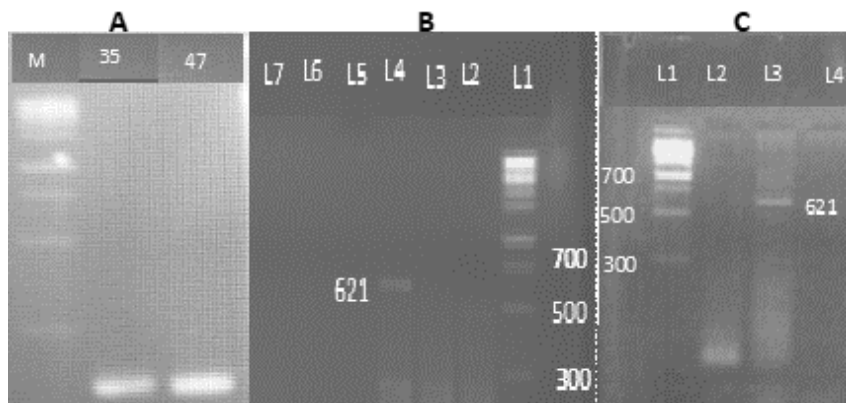
infected by multidrug-resistant bacteria (25) It was reported that there are interrelations between old age and antibiotic resistance (26). In the current study, 22% of isolates have NDM gene while no isolate has KPC, IMP gene. The results of another study revealed IMP gene in 9% of isolates. (21). Enterobacteriaceae with NDM-1 carbapenemases are extremely challenging to numerous anti-microbial classes. A study conducted by (27) showed the broad non-prescribed utilization of anti-microbials in India causing the emergence of NDM-1 which was probably going to deteriorate with the passage of time. Most NDM-1 positive plasmids are rapidly transferable. The quick development of NDM-1 is of incredible concern that couple of new drugs for Gram-negative are in the pharmaceutical pipeline and none are dynamic aligned with NDM-1 makers (28).

**Table No. 2: Resistance Pattern of *K. pneumoniae* Isolates Against Different Antibiotics**

Sr. No.	Antibiotics	Male	Female	Resistance %
		Sensitive / Resistant	Sensitive / Resistant	
1	AMC	7/14	16/13	54%
2	AMP	8/13	14/15	56%
3	FEP	7/14	16/13	54%
4	CTX	11/10	19/10	40%
5	CFM	9/12	14/15	54%
6	CZA	13/8	24/5	26%
7	CEC/CXM	8/13	15/14	54%
8	IMP/MEM	15/6	26/3	18%
9	AMK	15/6	28/1	14%
10	GEN/TOB	14/7	23/6	26%
11	DOX/NAL	14/7	23/6	26%
12	CIP/LEX	12/9	16/13	44%
13	NOR/OFX/MOX	12/9	16/13	44%
14	SXT	9/10	18/13	46%
15	FOS/TIC	9/10	18/13	46%
16	CST	20/0	30/0	0%
17	TGC/and TMP-SMX	13/8	23/6	28%
18	MXF/TZB	13/8	23/6	28%

**Table No. 3:** Relationship between the presence of virulent genes in *K. pneumoniae* with the Gender.

Gene	KPC		NDM		IMP	
	+ve	-ve	+ve	-ve	+ve	-ve
Male	0 (0%)	15 (36.58%)	1 (11.11%)	15(36.58%)	0 (0%)	15 (36.58%)
Female	0 (0%)	26 (63.41%)	1 (11.11%)	26(63.41%)	0 (0%)	26 (63.41%)

**Fig. No. 1:** Amplification of NDM, KPC and IMP Genes. (A) L1= 1000bp DNA Ladder. L2-L3=16Srna for the NDM gene. (B) L1=1000DNA Ladder. L4 positive samples for NDM gene. (C) L1 = DNA Ladder. L3 positive sample for NDM gene.

The presentation of NDM-1 in the UK is additionally and exceptionally stressing. It has incited arrival of a National Resistance Alert notice by the Department of Health on counsel of Health Protection Agency (29). The prospective for more extensive global spread of NDM-1-encoding plasmids to trigger up epidemics around the world, are apparent and alarming.

### CONCLUSION

The emergence of Multidrug-resistant (MDR) *Klebsiella* is inevitable and is a major public health threat worldwide. In Pakistan, numerous hospitals have experienced outbreaks of infections caused by MDR *Klebsiella* and MRSA with virulence factors (29). The current study concluded that the virulent genes *NDM* have a significant role in antibiotic resistance. The co-existence of Multidrug-resistant (MDR) and the virulent gene is an alarming situation. It needs more research to find out the mechanism of co-existence between virulent genes and antibiotic-resistant genes.

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