

# NDM-1 and *rmtC*-Producing *Klebsiella pneumoniae* Isolates in Turkey

Tulin Guven Gokmen,<sup>1\*</sup> Togrul Nagiyev,<sup>2</sup> Melda Meral,<sup>2</sup> Cansu Onlen,<sup>2</sup> Farzad Heydari,<sup>2</sup> and Fatih Koksali<sup>2</sup>

<sup>1</sup>Department of Microbiology, Ceyhan Veterinary Faculty, Cukurova University, Adana, Turkey

<sup>2</sup>Department of Medical Microbiology, Faculty of Medicine, Cukurova University, Adana, Turkey

\*Corresponding author: Tulin Guven Gokmen, Department of Microbiology, Ceyhan Veterinary Faculty, Cukurova University, Adana, Turkey. Tel: +90-3223386060, E-mail: tulinguven01@hotmail.com

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## Abstract

**Background:** The resistance of aminoglycosides in strains that produce beta-lactamase can be developed through the multidrug resistant encoding genes carried by common plasmids. Recently, the association between 16S rRNA methyltransferase resistance and beta-lactamase enzymes carried by the same plasmids has drawn increased attention from researchers, particularly the association in aminoglycoside-resistant strains with a minimum inhibitory concentration (MIC) of  $\geq 256 \mu\text{g/mL}$ .

**Objectives:** We aimed to investigate the co-existence of 16S rRNA methyltransferase and beta-lactamase genes in multidrug resistant (MDR) *Klebsiella pneumoniae* strains isolated from clinical samples.

**Methods:** We determined the molecular mechanisms of aminoglycoside resistance and its relationship with resistance to carbapenem and beta-lactam group antibiotics in 40 extended-spectrum beta-lactamase (ESBL)-positive carbapenem- and aminoglycoside-resistant *K. pneumoniae* strains. Multidrug resistant *K. pneumoniae* was isolated from various clinical samples in the faculty of medicine of Cukurova University, Turkey. First, the resistance of aminoglycoside and beta-lactam antibiotics was phenotypically investigated using the Kirby-Bauer disk diffusion test, double disk synergy test, and modified Hodge test. The MIC values of aminoglycoside were determined using the agar dilution method. Polymerase chain reaction was performed to detect the carbapenemases, ESBL, and 16S rRNA methyltransferase genes. The results were confirmed by a sequence analysis.

**Results:** Twenty *K. pneumoniae* strains showed resistance to amikacin, and 40 were resistant to gentamicin. The MIC value was found to be  $> 512 \mu\text{g/mL}$  in five amikacin-resistant strains and  $> 128 \mu\text{g/mL}$  in 10 gentamicin-resistant isolates. The *rmtC* gene, a type of 16S rRNA methyltransferase, was amplified in four isolates (MIC amikacin:  $> 512 \mu\text{g/mL}$ , gentamicin:  $> 128 \mu\text{g/mL}$ ). Of these four isolates, three had the blaNDM-1 gene and all contained at least one ESBL gene.

**Conclusions:** This study demonstrated the co-existence of *rmtC* and bla<sub>NDM-1</sub> genes for the first time in Turkey. The spread of this resistant type should be monitored and limited through molecular surveillance.

**Keywords:** Beta-Lactamase NDM-1, *RmtC*, 16S rRNA -m7G methyltransferase, *Klebsiella pneumoniae*

## 1. Background

The resistance of major gram-negative bacteria, such as *Escherichia coli* and *Klebsiella*, against antibiotics of the beta-lactam group, especially carbapenem, is increasing. These bacteria isolated from infections in hospitals all over the world result in the failure of treatment (1).

The combined use of aminoglycoside and beta-lactam group antibiotics increases permeability by chelation of bacterial cell walls. However, a high level of resistance against aminoglycoside is evident in strains that are resistant to beta-lactam group antibiotics (2, 3). This high resistance needs to be investigated in detail, and the molecular mechanisms and epidemiological features of aminoglycoside resistance should be the focus. According to molecular studies conducted to determine the resistance

mechanisms in bacteria against aminoglycoside, the following three mechanisms are responsible for the resistance: enzymes that modify aminoglycoside, mutation in genes that transport aminoglycoside, and alteration in ribosomal target proteins (4). A new resistance mechanism was discovered in 2003. This mechanism suggests that 16S rRNA methyltransferase genes are transported with plasmids and leads to a high level of resistance to aminoglycoside. This mechanism was demonstrated in *Pseudomonas aeruginosa* isolates in Japan and in *Enterobacteriaceae* isolates in France (5, 6). 16S rRNA methyltransferase was reported to significantly increase the level of aminoglycoside resistance by methylation of the G1405 residue (minimum inhibitory concentration [MIC] is typically  $\geq 256 \mu\text{g/mL}$ ) (7).

16S rRNA methyltransferase enzymes are generally seen together with carbapenemases and extended-spectrum beta-lactamase (ESBL). The existence of 16S rRNA methyltransferase has been observed in isolates carrying the genes *bla<sub>NDM-1</sub>*, *bla<sub>KPC-2</sub>*, *bla<sub>VEB</sub>*, *bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, and *bla<sub>OXA</sub>*. This outcome can be attributed to beta-lactamase and methyltransferase enzymes being located on the same plasmid (8-11).

## 2. Objectives

This study aimed to detect the co-existence of 16S rRNA methyltransferase enzymes and beta-lactamase genes in multidrug resistant (MDR) *Klebsiella pneumoniae* strains isolated from clinical samples in healthcare-associated infections.

## 3. Methods

A total of 228 clinical samples (i.e., blood, urine, bronchoalveolar lavage, sputum, and surgical wound) were collected from different clinics of the Medical Faculty of Balçali hospital at Cukurova University, Turkey, between August and December 2014. All the laboratory tests were performed at the laboratory of the Center and Medical microbiology department of the university. The samples were cultured on blood agar and Endo agar plates (Merck, Germany) at 37°C for 24 hours. The isolates were first evaluated by Gram staining to examine the morphology of colonies and biochemical test characteristics. Then, the bacterial species were identified using the VITEK-2 automated identification system (Biomérieux, Basingstoke, UK).

The resistance of beta-lactam antibiotics was investigated by the Kirby-Bauer disk diffusion (KBDD) test. The presence of ESBL was verified by the double disc synergy test, and carbapenem resistance was determined using the modified Hodge test. *Escherichia coli* ATCC 25922 was used as the control culture. To detect aminoglycoside resistance, amikacin (30 µg) and gentamicin (10 µg) sensitivities were examined through the KBDD test. Minimum inhibitory concentrations were determined by the agar dilution method on Mueller-Hinton agar plates according to the protocol recommended by the European committee on antimicrobial susceptibility testing (12).

In all isolates, DNA extraction was performed mechanically with a Mickle cell disruptor (The Mickle Lab. Engineering Co. Ltd., Gamshall, Surrey, UK). However, polymerase chain reaction (PCR) was performed only in isolates with an MIC value of > 512 µg/mL for amikacin and > 128 µg/mL for gentamicin to detect beta-lactamase genes and 16S rRNA methyltransferase genes. The multiplex PCR method

was used to determine the presence of *armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, and *rmtH* genes (13). In addition, a type-specific multiplex PCR protocol was used to determine the carbapenemase and ESBL genes (Table 1) (14-16). The PCR products were separated on 1.5% agarose gel and monitored using a Kodak UV transilluminator (Kodak, New York, USA). A sequence analysis was performed using the dye terminator cycle sequencing method and the ABI Prism Big dye terminator kit (Applied Biosystems, Foster City, USA). The assay was conducted according to the manufacturer's standard protocol. Data were collected on an ABI 310 automated fluorescence sequencer (Applied Biosystems). The types of 16S rRNA methyltransferase, carbapenemase, and ESBL genes were identified by comparing them with sequences from the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In all PCR procedures, distilled sterile water was used as the negative control. As no reference strains carrying *rmtC* were available, a positive control could not be used. Nevertheless, the sequences were verified by the DNA sequencing method.

## 4. Results

Forty isolates of *K. pneumoniae* were evaluated to determine the existence of and the relationship among carbapenemase, ESBL, and 16S rRNA methyltransferase genes. The strains were isolated from 4 blood, 15 urine, 7 bronchoalveolar lavage, 12 sputum, and 2 surgical wound samples. Twenty *K. pneumoniae* strains were resistant to amikacin, and 40 were resistant to gentamicin. The MIC values of amikacin and gentamicin were found to be > 512 µg/mL in five isolates and > 128 µg/mL in 10 isolates, respectively (Table 2).

PCR was performed only on five isolates with an MIC value of > 512 µg/mL for amikacin and > 128 µg/mL for gentamicin. A specific DNA fragment with a length of 246 bp was amplified on the *rmtC* gene in four isolates with the above-mentioned MIC values. Of these four isolates, three contained the *bla<sub>NDM-1</sub>* gene and all were found to have at least one ESBL gene (Table 3). A sequence analysis was performed to confirm the PCR results.

## 5. Discussion

Combination antibiotic therapy has been shown to have synergistic effects in vitro and in vivo. This treatment alternative is usually preferred in clinics to prevent the development of resistance without requiring a toxic dose or widening the antibacterial spectrum (17). In particular, carbapenem and aminoglycoside are used in

**Table 1.** PCR Primers Used in the Study (13, 14, 16)

Multiplex Primers	Gene	Forward/Reverse	Sequences (5'-3')	Product Size	
TEM, SHV and OXA-1-like	TEM	F	CATTTCGGTGTCCCTTATTC	800 bp	
		R	CGTTCATCCATAGTGCCTGAC		
	SHV	F	AGCCGCTTGACAAATTAAC	713 bp	
		R	ATCCCGCAGATAAATCACCAC		
	OXA-1 like	F	GGCACCAGATTCACTTCAAG	564 bp	
		R	GACCCCAAGTTCTCTGTAAGTG		
CTX-M group 1, group 2 and group 9	CTX-M group 1	F	TTAGGAARTGTGCCGTGYab	688 bp	
		R	CGATATCGTTGGTGGTRCCATb		
	CTX-M group 2	F	CGTTAACGGCAGATGAC	404 bp	
		R	CGATATCGTTGGTGGTRCCATb		
	CTX-M group 9	F	TCAAAGCTGCCGATCTGGT	561 bp	
		R	TGATTCTCGCCGCTGAAG		
CTX-M group 8/25	CTX-M-8, 25, 26, 39, 40, 41	F	AACRCRCAGACGGCTACb	326 bp	
		R	TGAGCCCGAAGGTGYATb		
VEB, PER and GES	GES	F	AGTCGGCTAGACCGGAAAG	399 bp	
		R	TTTGCCGTGCTCAGGAT		
	PER	F	GTCGCGATAATGAAGCGT	520 bp	
		R	TTCGGCTTGACTCGGCTGA		
	VEB	F	CATTCCCGATGCAAAGCGT	648 bp	
		R	CGAAGTTCTTTGGACTCTG		
SME,IMI	SME	F	GAGGAAGACTTTGATGGGAGGAT	334 bp	
		R	TCCCCTCAGGACCCCAAG		
	IMI	F	GGTGCTACGCTTTAGACACTGGCTC	536 bp	
		R	GCACGAATACGGCTGCACCGG		
OXA-48-like	OXA-48-like	F	GCTTGATCGCCCTCGATT	281 bp	
		R	GATTTGCTCCGTGGCCGAAA		
IMP, VIM and KPC	IMP	F	TTGACACTCATTACDgb	139 bp	
		R	GATYGAGAATTAAGCCACYCTb		
	VIM	F	GATGGTGTGTGGTCGATA	390 bp	
		R	CGAATGGCAGCACAG		
	KPC	F	CATTCAAGGGCTTCTTGCTGC	538 bp	
		R	ACGACGGCATAGTCATTGC		
GIM-1, SIM-1, NDM-1 and SPM-1	GIM	F	CGTTGCCAGCTTTAGCTCAGG	279 bp	
		R	GCAACTGTATACCAGCAGTCCG		
	SIM	F	TTGCGGAAGAAGCCAGCCAG	613 bp	
		R	GCG TCT CCG ATT TCA CTG TGG C		
	NDM	F	CCC GGC CAC ACC AGT GAC A	129 bp	
		R	GTA GTG CTC AGT GTC GGC AT		
	SPM	F	GGG TGG CTA AGA CTA TGA AGC C	447 bp	
		R	GCC GCC GAG CTG AAT CGG		
rmtA, rmtC, rmtD, rmtG, rmtH	rmtA	F	AAACTATTCCGATGGTTC	88 bp	
		R	TCATGTACACAAGCTCTTCC		
	rmtC	F	CAGGGGTTCACAAGT	246 bp	
		R	AGAGTATATAGCTTGAACATAAGTAGA		
	rmtD	F	TCGTTTCAGCACGTAAMACA	652 bp	
		R	CAGCGGAAATTCAAAAAGG		
	rmtG	F	ACGGAATGCCGCGGAAGTA	381 bp	
		R	TCTCCGCAAGCAGATCGCCG		
	rmtH	F	ATGACCATTGAACAGGCAGC	464 bp	
		R	AGGGCAAAGGTAATCCCA		
	armA, npmA, rmtB, rmtE, rmtF	armA	F	ATTTAGATTTTGGTTGTGGC	101 bp
			R	ATCTCAGCTCTATCAATATCG	
npmA		F	GGGCTATCTAATGGTG	229 bp	
		R	TTTTTATTCCGCTTCTCGT		
rmtB		F	ACTTTIACAATCCCTCAATAC	171 bp	
		R	AAGTATATAAGTCTGTCCG		
rmtE		F	GATGCCGTGCTGTACGCCG	446 bp	
		R	ACGTGAACCCACGAGTCTGC		
rmtF		F	CGATCCTACTGGGCTCCAT	314 bp	
		R	GGCATAGTGCTTTCCATGC		

combination prior to colistin and tigecycline in the treatment of infections of *Enterobacteriaceae* isolates that produce carbapenemase. For example, the combinations of

meropenem/imipenem and amikacin or doripenem and gentamicin have been suggested as an effective alternative treatment for *K. pneumoniae* strains that produce car-

**Table 2.** Distribution of Amikacin and Gentamicin MIC Values Among the *K. pneumoniae* Isolates

Amikacin MIC Value, $\mu\text{g/mL}$	Number of Isolates	Gentamicin MIC Value, $\mu\text{g/mL}$	Number of Isolates
> 512	5	> 128	10
128	5	128	10
64	5	64	4
32	1	32	8
16	4	16	2
-	-	8	2
-	-	4	4
<b>Total</b>	<b>20</b>	<b>Total</b>	<b>40</b>

**Table 3.** Co-Existence of ESBL and 16S rRNA Methyltransferase Genes Among the *K. pneumoniae* Isolates (BAL: Bronchoalveolar Lavage)

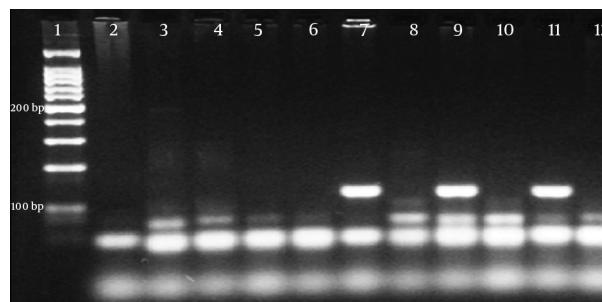
Isolates	Amikacin MIC value, $\mu\text{g/mL}$	Gentamicin MIC Value, $\mu\text{g/mL}$	16S Rrna Methyltransferase Gene	Carbapenemase Genes	ESBL Genes
1 (Urine)	> 512	> 128	rmtC	NDM-1	SHV
2 (Urine)	> 512	> 128	rmtC	NDM-1, OXA-48	TEM,SHV
3 (Blood)	> 512	> 128	rmtC	OXA-48	TEM
4 (BAL)	> 512	> 128	rmtC	NDM-1,OXA-48	TEM,SHV
5 (BAL)	> 512	> 128	-	OXA-48	SHV

**Figure 1.** rmtC-PCR Products



Lane 1, 50 bp DNA ladder; lane 4, sample 1; lane 7, sample 2; lane 13, sample 3; lane 16, sample 4 (246 bp); lane 9, negative control.

**Figure 2.** NDM-1-PCR Products



Lane 1, 100 bp DNA ladder; lane 2, negative control, lane 7, sample 1; lane 9, sample 2; lane 11, sample 4 (129 bp); lane 9, negative control.

carbapenemase (18, 19).

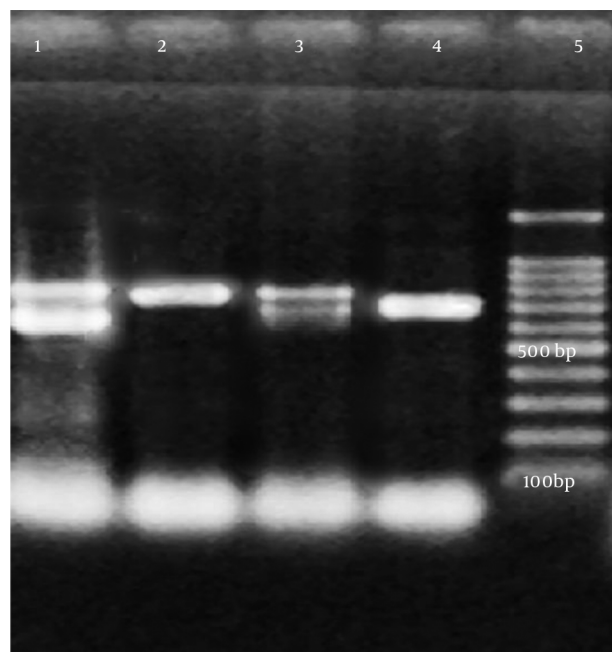
In recent years, plasmids that transport ESBL and carbapenemase enzymes have been found to carry 16S rRNA methyltransferase aminoglycoside-resistant genes. Thus, aminoglycoside and beta-lactam antibiotic combinations are no longer considered clinically effective. This finding was verified by Wu et al., who detected the *armA* methyltransferase gene on a plasmid that codes the *K. pneumoniae* carbapenemase enzyme (20).

Despite having low prevalence in different types of bacteria, the 16S rRNA methyltransferase gene is globally spreading because of the plasmids that transport carbapenemase and ESBL genes among gram-negative bacilli. In Taiwan, the *rmtB* and *armA* genes were identified in *E. coli*

and *K. pneumoniae* isolates with a high level of amikacin resistance, and the prevalence of *armA* was found to be higher than that of *rmtB* (7). In Japan, *rmtA*, *rmtB*, *npmA*, and *armA* were detected in gram-negative isolates with an amikacin MIC value of over 512 mg/L (21). In Korea, the co-existence of *rmtE* and CMY-2 cephalosporinase was reported (22). In China, *armA* and *rmtB* genes were found in gram-negative bacteria isolates with the amikacin MIC value being consistently over 512 mg/L (23).

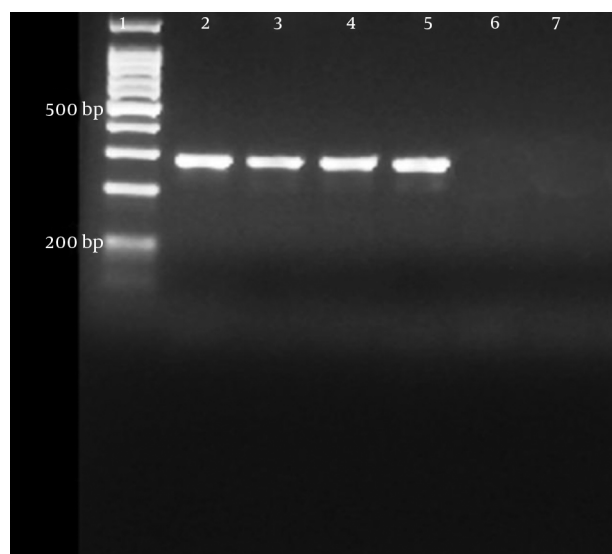
In Saudi Arabia, the relationship between ESBL and 16S rRNA methyltransferase genes was investigated, and the

**Figure 3.** TEM and SHV-PCR Products



Lane 5, 100 bp DNA ladder; lane 4, sample 1; lane 3, sample 2; lane 2, sample 3; lane 1, sample 4 (TEM: 800 bp, SHV: 713 bp).

**Figure 4.** OXA-48-PCR Products



Lane 1, 100 bp DNA ladder; lane 2, sample 2; lane 3, sample 3; lane 4, sample 4; lane 5, sample 5; lane 6, negative control.

*rmtB*, *rmtC*, and *armA* genes were detected in the same isolates (24). Fritsche et al. analyzed gram-negative bacteria

isolates from North America, Latin America, and Europe and found that the *armA*, *rmtB*, and *rmtD* genes were resistant to aminoglycosides in isolates with amikacin MIC values of over 128  $\mu\text{g/L}$  (25). The existence of the *rmtF* methyltransferase gene was reported in Nepal, the United States, India, and England (26). The *rmtH* gene was determined in a *K. pneumoniae* strain isolated from an American soldier who was wounded in Iraq in 2006 (27). In Turkey, the *rmtB* gene was shown in a *K. pneumoniae* isolate that was aminoglycoside resistant (28, 29). In another study, 16S rRNA methyltransferase resistance was not found in any of the 59 aminoglycoside-resistant clinical isolates that were evaluated over a five-year period (30).

In the current study, the *rmtC* gene was detected in four isolates with an MIC value of  $> 512 \mu\text{g/mL}$  for amikacin and  $> 128 \mu\text{g/mL}$  for gentamicin. We determined both NDM-1 and *rmtC* genes in three isolates with a high level of aminoglycoside resistance. We found at least one ESBL gene in all four isolates that contained the *rmtC* gene. The co-existence of the NDM-1 and *rmtC* genes was previously observed in *K. pneumoniae* strains isolated from clinical samples in Australia, Nepal, Kenya, and Bangladesh. However, to the best of our knowledge, this study is the first report conducted in Turkey showing the presence of the *rmtC* gene and the co-existence of NDM-1 and *rmtC* resistance genes among clinical isolates.

The combination of beta-lactam and aminoglycoside is an important treatment alternative for gram-negative bacterial infections, but it has lost its clinical significance because of 16S rRNA methyltransferase and other aminoglycoside-resistant mechanisms. 16S rRNA methyltransferase-type resistance has low prevalence but spreads easily in plasmids that transport beta-lactamase genes such as NDM-1. This resistance can spread across a wide geographic region in Turkey and in other parts of the world. Further studies are needed to determine the mechanisms of 16S rRNA methyltransferase and carbapenemase resistance. The spread of these types of resistances should be monitored using molecular analyses.

**Footnotes**

**Authors' Contribution:** Study concept and design, Fatih Koksak and Tulin Guven Gokmen; acquisition of data, Tulin Guven Gokmen, Melda Meral, Cansu Onlen, and Farzad Heydari; analysis and interpretation of data, Tulin Guven Gokmen, Togrul Nagiyev, and Fatih Koksak; drafting of the manuscript, Tulin Guven Gokmen; critical revision of the manuscript, Tulin Guven Gokmen and Fatih Koksak; administrative, technical, and material support, Fatih Koksak. Study supervision, Fatih Koksak and Tulin Guven Gokmen.



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