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Detection of 16S Ribosomal RNA Methylation in Extended-Spectrum β-lactamase-Producing *Klebsiella Pneumoniae* Clinical Isolates from Baghdad Hospitals

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Abstract: Fifty-eight Clinical isolates of *Klebsiella pneumoniae* were collected from patients at Al-Kindey teaching hospital, Al-Kadhymia teaching hospital, Ibn-Albalady hospital and Al-Imam-Ali hospital in Baghdad from Urinary tract infection (UTI) patient. These isolates were diagnosed using different morphological and biochemical test followed by the complementary API 20E, isolates all screened for antibiotic resistance by rapid detection of β-lactamases production method and their minimum inhibitory concentrations(MICs) were conducted by polymerase chain reaction assay. Detection of $bla_{CTX\ -M}$ gene was performed and highly resistant isolates (n=17) were selected to determine aminoglycoside resistance mediated by methylation 16S rRNA that combined with the detection of $bla_{CTX\ -M}$ gene responsible for Extended Spectrum β-lactamase (ESβLs) production. Seven 16S rRNA methylase genes were amplified, the *ArmA*, *RmtA*, *RmtB*, *RmtC*, *RmtD*, *RmtF* and *npmA* beside amplifying $bla_{CTX\ -M}$ gene. This study explained the physically link between $bla_{CTX\text{-}M}$ gene (ESβLs) production among *Klebsiella pneumoniae* in correlation with 16S rRNA methylation.

Key words: *Klebsiella pneumoniae*, ESβLs, $bla_{CTX\text{-}M}$ gene, 16 rRNA methylation genes.

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Introduction

Aminoglycosides, along with β-lactams, is currently one of the key classes of antimicrobial agents in the treatment of broad range of life-threatening infections caused by Gram-negative bacteria. Mechanisms of resistance to aminoglycosides include enzymatic modification of the drugs, modification of the aminoglycoside-binding site, decreased permeability across the bacterial outer membranes, and augmented efflux. Among them, production of acquired 16S rRNA methyltransferase is the most worrisome since it inhibited the activity of all aminoglycosides (1). In Iraq, aminoglycosides were commonly used for treating severe infections caused by Gram-negative bacteria. As a result, multiple resistance determinants to these antimicrobial agents have emerged in various pathogenic microbes. Post- transcriptional methylation of the 16SrRNA has been reported, and these results in high-level resistance to aminoglycoside antibiotics (2). At present, ten 16S rRNA methylase genes have been identified, including *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH*, and

npmA, which are capable of conferring high levels of resistance to most clinically important aminoglycosides, including amikacin, gentamicin, kanamycin, and tobramycin. (1, 2, 3,4, 5,6,7,8, 9).
RmtB and *ArmA* are the most frequently identified methylases in Enterobacteriaceae isolated in East Asia, Europe, and South America. (10, 11,12,13,14). The six methylase enzymes expressed from the six genes have been described to be carried on plasmid and the pathogenic bacteria producing such enzymes have the ability to resist all aminoglycoside group. (11,15).
Associations between 16S rRNA methylase and extended-spectrum β-lactamase (ESβLs) production specially bla_{CTX-M} group have been reported (11,14,15).
The aim of this study was to identify the prevalence of 16S rRNA methylase genes (*ArmA, RmtA, RmtB, RmtC, RmtD, RmtF* and *NpmA*) among ESβL-producing *K. pneumoniae* isolates harbouring bla_{CTX-M} obtained from patients presented with urinary tract infection (UTI).

Materials and Methods

Clinical Background

Fifty-eight clinical isolates of *Klebsiella pneumoniae* were isolated from patients at Al-Kindey teaching hospital, Al-Kadhymia teaching hospital, Ibn-Albalady hospital and Alemam-Ali hospital in Baghdad during a period between July 2011 and December 2011. They were obtained from midstream urine from patients suffering from (UTI). Bacterial diagnosis including morphological and biochemical tests were done according to Atlas *et al.* (16) followed by the complementary API 20E test.

Rapid Detection of β-lactamases Production

The detection of β-lactamases production was performed using Rapid ESβL detection kit (MAST Group, UK). This kit includes four tests: preliminary screening kit, Metallo-β-lactamases, ESβLs confirmation and AmpC detection. The test was performed according to the procedure suggested by the manufacturing company: bacterial isolates were cultured on Muller Hinton agar medium with cefotaxime 30µg disc. The resisted isolates were submitted to ESβLs production test. One drop of test substrate (approximately 20µl) was dispensed onto the filter pad of the strip. The test substrate was added to the strip immediately before testing. Using a loop, one colony was picked up and spread on the filter pad of the test strip any change in color observed around the streaked line was considered a positive result. The test strip was observed after 2 to 15 minutes at room temperature, and the result was read after 15 minutes.

Antibiotics Susceptibility Assay

All isolates were tested for MIC according to the CLSI (17) two fold agar dilution method were used (18,19), using four types of aminoglycoside antibiotic including: amikacin, kanamycin, gentamicin, paromomycin. *Escherichia coli* ATCC 25922 were used as quality control strain(Central Public Health Laboratory, Baghdad).

Detection of Methylase and ESβLs Genes Using PCR

Plasmid DNA was extracted according to alkaline lysis method Crosa *et al.* (20) from overnight bacterial growth. The plasmid DNA was used as a DNA template for the PCR technique. The *armA, rmtA, rmtB, armC, rmtD, rmtF, npmA* and bla_{CTX-M} genes were detected by PCR using specific primers listed in table 1. PCR products were electrophoresed in 1.5% agarose gel and visualized under UV light. (Master Mix and 100 bp DNA molecular ladder which used in this study from KAPA(south Africa))(21).

Table 1: Primers for detection of 16S rRNA methylation genes

PCR target	Primer Name	Sequence (5′→3′)	Amplicon size, base pairs	Reference
bla_{CTX-M}	*CTX-M-F*	CGCTTTGCGATGTGCAG	550	(37)
	CTX-M-R	ACCGCGATATCGTTGGT		
RmtA	rmtA-F	CTAGCGTCC ATCCTTTCCTC	635	(34)
	rmtA-R	TTGCTTCCA TGCCCTTGCC		
RmtB	rmtB-F	CCC AAA CAG ACC GTA GAGGC	584	(15)
	rmtB-R	CTC AAA CTC GGC GGG CAAGC		
RmtC	rmtC-F	CGA AGA AGT AAC AGC CAA AG	711	(4)
	rmtC-R	ATC CCA ACA TCT CTC CCA CT		
RmtD	rmtD-F	TCAAAAAGGAAAAGGACGTG	500	(38)
	rmtD-R	CGATGCGACGATCCATTC		
RmtF	rmtF –F	GCGATACAGAAAACCGAAGG	453	(39)
	rmtF -R	GGCAGGAGCTTCATCAGAA		
ArmA	armA-F	CCGAAATGACAGTTCCTATC	846	(40)
	armA-R	GAAAATGAGTGCCTTGGAGG		
NpmA	npmA-F	CTCAAAGGAACAAAGACGG	641	(38)
	npmA-R	GAAACATGGCCAGAAACTC		

Results

A total 58 of *Klebsiella pneumoniae* clinical isolates, were collected from Baghdad hospitals, have been studied. At first, the resistance mechanisms are identified by using of specific inhibitors on the color-coded test strips i.e. ESβL (Clavulanic Acid), MβL (EDTA and Mercaptoacetic Acid) and AmpC (Boronic Acid). It is easy to perform highly specific, different types of enzymes could be detected in the same run. The findings from antimicrobial tests showed that bla_{CTX-M} -positive isolates were revealed 17(100%) resistance to ESβLs, 8(47%) were resistance to MβL and 7(41.1%) resistance for AmpC of a total of 17 isolates. Whereas bla_{CTX-M} -negative revealed 36(100%) were resistance to ESβL, 27(75%) and 3(8.3%) were resistance for MβL and AmpC respectively Table (2). Figure 1 depicts

the amplified *bla*$_{CTX-M}$ gene in *K. pneumoniae* isolates. This study has also revealed that isolates were aminoglycoside resistant to commercially aminoglycoside antibiotic the percentage of resistance were 17(100%) to Amikacin, Gentamicin, Kanamycin and Paromomycin by phenotypic confirmatory test. Whereas *bla*$_{CTX-M}$ -negative isolates their resistance were between susceptible, intermediate and resistant to Amikacin, Gentamicin, Kanamicin and Paromomycin (Table 3). This study showed that all *bla*$_{CTX-M}$ negative isolates were sensitive to amikacin therefore; the amikacin antibiotic was the best drug of choice for *K. pneumoniae*.

Table 2: Frequency of ESBLs-producing strains and susceptibility pattern

β-lactemase type	*bla*$_{CTX-M}$ -positive (n = 17)		*bla*$_{CTX-M}$ -negative (n = 36)	
	Resistant n (%)	Susceptible n (%)	Resistant n (%)	Susceptible n (%)
ESBLs	17(100)	0	36(100)	0
MBL	8(47)	9(52.9)	27(75)	9(25)
AmpC	7(41.1)	10(58.8)	3(8.3)	33(91.7)

n= number

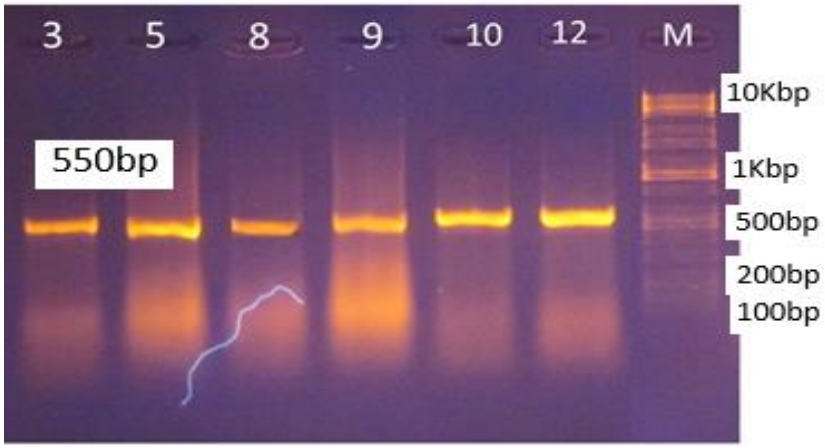

Figure 1: Gel electrophoresis (1% agarose, 7 V/cm for 90min) of *bla*$_{CTX-M}$ gene in *K. pneumoniae* isolates using plasmid DNA as a template. lane M :100 bp DNA ladder , lanes (3,5,8,9,10,12) positive results for blaCTX-M gene with 550bp amplicon

All the 17 resistant isolates were screened by PCR for the detection of 16S rRNA methylase-encoding genes (*armA, rmtA, rmtB, rmtC, rmtD, rmtF*

and *npmA*). High levels of resistance to aminoglycosides conferred by plasmid-mediated mechanisms corresponding to 16S rRNA methylation, six genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtF* and *npmA*) have been shown in figure 2. The MICs results for four aminoglycoside antibiotics (Amikacin, kanamycin, Gentamicin and Paromomycin) were shown (100%) resistance. Isolates was characterized as resistant in comparison with CLSI (17). All the 17 *K. pneumoniae* isolates revealed resistance to Gentamicin (MIC ranged between 128-1024 µg /ml), Kanamycin (MIC ranged from 256-1024 µg /ml), Paromomycin (MIC ranged from 512-1024µg /ml) and Amikacin when (MIC ranged from 64-256 µg /ml) Table (4).

Table 3: Frequency of aminoglycoside resistance strains and susceptibility pattern to aminoglycoside antibiotic

Aminoglycoside	bla_{CTX-M} -positive (n = 17)			bla_{CTX-M} -negative (n = 36)		
	Resistant n(%)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)	Susceptible n (%)	Intermediate n (%)
AMK	17(100)	0	0	0	30(83.3)	6(16.7)
GEN	17(100)	0	0	35(97.2)	0	1(2.77)
KAN	17(100)	0	0	34(94.4)	0	2(5.5)
PMR	17(100)	0	0	34(94.4)	0	2(5.5)

R, resistant; **I**, intermediate; **S**, sensitive. **AK:**amikacin**, GN:**gentamicin**, K:**kanamycin**, P:** paromomycin

Table 4:MICs of antibiotics for clinical isolates producing 16S rRNA methylases

Clinical isolate	16S rRNA methylase or/and ESBLs	MIC (µg/ml)			
		AMK ≥64	GEN ≥16	KAN ≥64	PMR ≥16
K. pneumonia 3	*bla*$_{CTX-M}$ and *npmA*	128	512	1024	1024
K. pneumonia 5	*bla*$_{CTX-M}$	128	512	256	512
K. pneumonia 8	*bla*$_{CTX-M}$ and *rmtB*	128	512	256	512
K. pneumonia 9	*bla*$_{CTX-M}$, *rmtC, rmtD and rmtF*	128	1024	1024	1024
K. pneumonia 10	*bla*$_{CTX-M}$	128	256	512	512
K. pneumonia 12	*bla*$_{CTX-M}$, *rmtD* and *npmA*	128	512	512	512
K. pneumonia 17	*bla*$_{CTX-M}$	128	512	512	1024
K. pneumonia 18	*bla*$_{CTX-M}$, *rmtC* , *armA* and *npmA*	128	1024	1024	512
K. pneumonia 23	*bla*$_{CTX-M}$ and *rmtB*	128	512	256	512
K. pneumonia 24	*bla*$_{CTX-M}$	128	1024	1024	1024
K. pneumonia 28	*bla*$_{CTX-M}$, *rmtD and armA*	128	1024	512	512
K. pneumonia 32	*bla*$_{CTX-M,}$ *armA*	64	256	512	1024
K. pneumonia 35	*bla*$_{CTX-M}$	128	1024	1024	1024
K. pneumonia 36	*bla*$_{CTX-M}$, *rmtC* and *npmA*	256	1024	1024	1024
K. pneumonia 39	*bla*$_{CTX-M}$, *armA* and *npmA*	256	1024	512	1024
K. pneumonia 40	*bla*$_{CTX-M}$, *rmtC, armA* and *rmtA*	128	1024	1024	1024
K. pneumonia 51	*bla*$_{CTX-M}$ and *armA*	256	1024	1024	1024

Arm, Antibiotic-resistant marker abbreviations: **AMK**, amikacin; **GEN**, gentamicin; **KAN**, kanamycin; **PMR**; paromomycin

A total of 17 ESβL-producing *K. pneumoniae* clinical isolates harbored *bla*$_{CTX-M}$ gene, have been studied. All 17 isolates (100%) were resistant to aminoglycosides, screened by PCR for detection of 16S rRNA methylase-encoding genes *(armA, rmtA, rmtB, rmtC, rmtD, rmtF and npmA*). Surprisingly, only five isolates (3, 8, 23, 32, 51) have single gene of the 16S rRNA methylation genes combined with the presence of *bla*$_{CTX-M}$ gene, whereas six isolates have two genes of the 16S rRNA methylation genes (12, 18, 28, 36, 39, 40), and one isolate have three genes.

In addition, five isolates (5, 10, 17, 24, 35) were resistant to aminoglycoside but were negative for 16sRNA methylation genes.

Discussion

We aimed to study the clinical epidemiology of these emerging co-resistant isolates. The prevalence of antimicrobial resistance among microorganisms that cause UTI is increasing worldwide problem and a

major factor in selecting antibiotics for treatment (22). The result comparatively resemble literature available. Co-resistance with β-lactams are frequently seen in ESβL-producing gram negative bacteria particularly to aminoglycosides, beside that genes encoding ESβLs are typically carried on the same self-transferable plasmids that often carry other determinants of antibiotic resistance (23,24). The organisms that produce 16SrRNA methylase are often multidrug resistant, especially against broad-spectrum β-lactams via production of ESβLs or metallo-β-lactamases. 2-tiered approach, consisting of phenotypic tests followed by PCR confirmation is recommended for detection of 16SrRNA methylase-mediated resistance.

From noticing the results of *K. pneumoniae* isolates, it's clear that the high-level of resistance (100%) was toward Amikacin, gentamicin, kanamycin and paromomycin when the MIC of some isolates reached 1024 µg /ml (gentamicin, kanamycin and paromomycin) while the break point for these antibiotic is only 16 µg /ml except for kanamycin which is 64 µg /ml. It could be said that the best activity was to amikacin when the highest titer of MIC was 256 µg /ml as a maximum rate for some isolates while the break point for this antibiotic is 64 µg /ml.

The literature showed the values of MIC for gentamicin against *E.coli* isolates were 0.5-256 µg/ml, these values are much lower than Risberg findings, (25) while Yamane *et al*. (26) reported that the MIC values reached to 1024 µg/ml, thus it agreed with this study. For paromomycin MIC was between 32-1024 µg/ml, the result of this study coincides with the results of Fritsche *et al*. (27). In Brazil the MIC for Amikacin was (16-128) µg/ml and 32-256 µg/ml by Fritsche *et al*. (27) and Gonza′les-Zorn *et al.* (28)

The range of MIC values for kanamycin and gentamicin were between 32-1024 µg/ml and 16-1024 µg/ml respectively which is coincide with the current study (29). Gad *et al*. (29) and Lim *et al*. (30) they reported that the rang of MIC to amikacin was between (64-128) µg/ml which is convenient with the current study.

All kind of methylation genes were detected in the local *K. pneumoniae* isolates. One of the interesting results in the current study is that one isolates of *K. pneumoniae* no. 9 harbored the three-methylation genes alongside with bla_{CTX-M} gene table 4. Only one methylation gene appeared in five isolates (no.3, 8, 23, 32, 51) in addition to bla_{CTX-M} gene. In addition there are five isolates harbourd only bla_{CTX-M} gene because the resistance for aminoglycoside may be related to the mutation in 23S rRNA, efflux mechanisms, aminoglycoside modifying enzymes (AMEs) and impermeability of the cell wall may cause reduced susceptibility to aminoglycosides and the most prevalent was efflux mechanisms according to Gallego and Towner (31), Nemec *et al*. (32), Shaw *et al*. (33). In this study, it seems to be an association between ESβL-coding genes and 16S rRNA methylases is taken a place as it is summarized in table (4) which shows the correlation between bla_{CTX-M} gene and 16S rRNA methylase genes, since these genes are sometimes located on the same conjugative plasmid (34). Other finding which is fixed in the current study, that two or more 16S rRNA methylation genes were located on a same plasmid

belonging to bla_{CTX-M} gene (*K. pneumoniae* no.12, 18 28, 36, 39, and 40), especially *K. pneumoniae* no.9 that contained three 16S rRNA methylase genes and bla_{CTX-M} gene.

The responsible genes are mostly located on transposons within transferable plasmids, which provides them with the potential to spread horizontally and may in part explain the already worldwide distribution of this resistance mechanism, some of these isolates have been found to co-produce ESβL or MβL, contributing to their multidrug-resistant phenotypes. (4,24,35) This study underlines the co-association of 16S rRNA methylase and ESβL-encoding genes linked together resulting in the multidrug-resistant for both of *K. pneumoniae,* which is considered the first report in Iraq and may become a clinical problem. Worryingly, an increase in the prevalence of ESβL, led to the prevalence of aminoglycoside resistance is likely to increase in the coming years. In Poland *K. pneumoniae* epidemic strain that coproduced carbapenemase and 16S rRNA methylase ArmA has emerged were found to carry the blaKPC-2 and armA genes on plasmids (36). Acquired MβL genes are located on integron that reside on mobile genetic elements such as plasmids or transposons, thus, enabling widespread dissemination. (35)

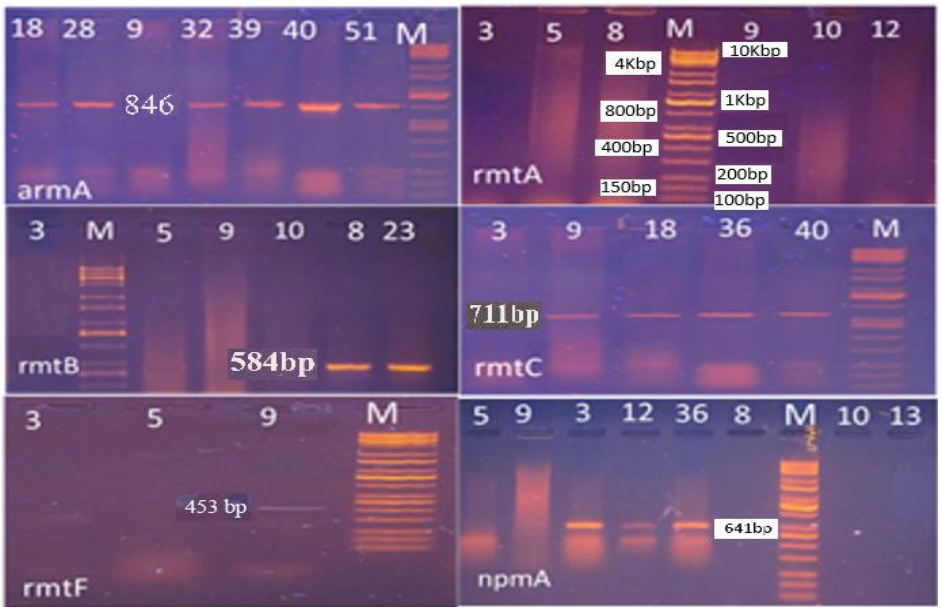

Figure 2: PCR amplification of *rmtF, armA, npmA, rmtA, rmtB and rmtC* genes

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