

## Detection of 16S rRNA Methylases and Co-Resistance with $\beta$ -lactams among *Klebsiella pneumoniae* Isolates from Iraqi Patients

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### Abstract:

Out of 150 clinical samples, 50 isolates of *Klebsiella pneumoniae* were identified according to morphological and biochemical properties. These isolates were collected from different clinical samples, including 15 (30%) urine, 12 (24%) blood, 9 (18%) sputum, 9 (18%) wound, and 5 (10%) burn. The minimum inhibitory concentrations (MICs) assay revealed that 25 (50%) of isolates were resistant to gentamicin ( $\geq 16 \mu\text{g/ml}$ ), 22 (44%) of isolates were resistant to amikacin ( $\geq 64 \mu\text{g/ml}$ ), 21 (42%) of isolates were resistant to ertapenem ( $\geq 8 \mu\text{g/ml}$ ), 18 (36%) of isolates were resistant to imipenem ( $4- \geq 16 \mu\text{g/ml}$ ), 43 (86%) of isolates were resistant to ceftriaxone ( $4- \geq 64 \mu\text{g/ml}$ ), 42 (84%) of isolates were resistant to ceftazidime ( $16-64 \mu\text{g/ml}$ ), and 40 (80%) of isolates were resistant to cefepime ( $4- \geq 16 \mu\text{g/ml}$ ). Co-Resistance for both  $\beta$ -lactams and aminoglycosides were detected among 25 (50%) of *K. pneumoniae* isolates. The extended spectrum beta-lactamases (ESBLs) were detected among 25 (50%) of *K. pneumoniae* isolates. Screening of 16S rRNA methylases encoding genes revealed that *armA* was found in 5 (10%) of *K. pneumoniae* isolates, whereas *rmtB* was not found among *K. pneumoniae* isolates. DNA sequencing of *armA* revealed that the presence of missense mutations in which affected in the translation of protein by substitutions of amino acids, leading to increase the resistance values of MICs for gentamicin and amikacin. These variants were registered in NCBI at the accession number LC373258. The phylogenetic tree of *armA* variants showed a slight deviation of these variants from *K. pneumoniae* species.

**Key words:** ESBLs, *Klebsiella pneumoniae*, Phylogenetic tree, 16S rRNA methylases.

### Introduction:

*Klebsiella pneumoniae* is a member of Enterobacteriaceae, rod shaped, Gram negative, non motile, lactose fermentor, and facultative anaerobe with a mucoid capsule (1). It is an opportunistic pathogen that infects immunocompromised patients or who are suffered from other infections (2). The opportunistic *K. pneumoniae* can colonize mucosal surfaces of human. This colonization can progress to serious community acquired or nosocomial infections, including urinary tract infections (UTIs), pneumonia, bacteremia, liver abscesses, endogenous endophthalmitis, and cystitis (3).

At last decade, *K.pneumoniae* evolves resistance mechanisms against most common usage antibiotics, including aminoglycosides. This leads to limit the options of clinical treatment. Consequently, that leads to high rates of morbidity and mortality worldwide (4).

Aminoglycosides are bactericidal and moderate spectrum antibiotics that widely used to

treat life threatening infections caused by *K. pneumoniae*. They act by binding to the highly conserved A site, a part of the 16S rRNA within the small subunit 30S of bacterial ribosome, leading to block protein synthesis (5).

*K.pneumoniae* uses several mechanisms for inhibiting the activity of aminoglycosides. The Aminoglycosides Modifying Enzymes (AMEs) are the most common mechanism employed by *K.pneumoniae* to interfere with the action of aminoglycosides through N-acetylation, O-nucleotidylation or O-phosphorylation of these drugs (6). The other resistance mechanisms against aminoglycosides are less common, including alternations of cell permeability and modification of target through mutations in ribosomal proteins or in 16S rRNA. These lead to reduce the uptake of aminoglycosides and loss their actively action (7).

16S rRNA methylases are recently emerged among *K.pneumoniae* isolates as new resistance mechanisms that act by preventing aminoglycosides to bind to 16S rRNA, the target of aminoglycosides (8). These enzymes confer high resistance against almost all common aminoglycosides, including

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gentamicin, amikacin, tobramycin, kanamycin, isepamicin, arbekacin, and even against plazomicin, the most recent developed drug of aminoglycosides. In contrast, AMEs have a narrow spectrum of activity against aminoglycosides (6).

The clinically important *K.pneumoniae* is found to harbor three families of 16S rRNA methylases including, Arm, Rmt, and NpmA which are mainly carried on plasmids (7). The gene *armA* was first reported on a plasmid of *K.pneumoniae* from France and then recognized on a chromosome of this species (9). The location of 16S rRNA methylases encoding genes on plasmids enhances the rapid dissemination of these genes to other clones or species of Enterobacteriaceae and even of non-Enterobacteriaceae through horizontal gene transfer (HGT) (5).

16S rRNA methylases often act synergistically with  $\beta$ -lactamases due to the presence of genes encoding for 16S rRNA methylases (*armA*) and  $\beta$ -lactamases (*bla<sub>CTX-M</sub>* or *bla<sub>NDM</sub>*) on the same plasmid (10). This led to emergence of multidrug resistant (MDR) and extremely drug resistant (XDR) *K.pneumoniae* which have a great ability to cause and outbreak of serious infections due to their resistance for most common antibiotics in clinical usage (4).

This study aimed to investigate the prevalence of 16S rRNA methylases encoding genes *armA* and *rmtB* and co-resistance for aminoglycosides and  $\beta$ -lactams among clinical isolates of *K.pneumoniae*.

## Materials and Methods:

### Collection of Samples:

A total of 150 clinical samples (urine, blood, sputum, wounds, and burns) were collected from patients who were suffered from different cases, including UTIs, bacteremia, pneumonia, and burns and wounds infections from different hospitals in Baghdad. The collection of samples was done according to the instructions of the ethics committee at the Ministry of Health in Baghdad.

### Bacterial Isolation and Identification:

The bacteria were isolated from clinical samples by culturing on MacConkey agar, Blood

agar, Eosin Methylene Blue agar (Oxoid,UK), and CHROMagar Orientation (Pioneer,France). All culture media were incubated at 37°C for 24hrs (11). Then the bacterial isolates were identified by using biochemical assays, including oxidase, catalase, and IMVIC tests. The Vitek 2 Compact System (BioMerieux,France) was used for confirmation of identified bacteria (12).

### Minimum Inhibitory Concentrations (MICs):

The MICs were performed by using Vitek 2 Compact System against 7 antibiotics, including Amikacin, Gentamicin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, and Imipenem. The results were interpreted according to CLSI (13).

### Phenotypic Detection of Extended Spectrum Beta-Lactamases (ESBLs):

The ESBLs were detected by using Vitek 2 Compact System for all *K. pneumoniae* isolates (12).

### DNA Extraction:

The Wizard Genomic DNA Purification Kit (Promega,USA) was used to extract DNA from bacterial isolates according to the informations of manufacturing company. The concentrations and purity of DNA were measured by using Nanodrop (Biogroup,UK) (14).

### Determination of Genotyping of *armA* and *rmtB* Genes:

The extracted DNA from *K. pneumoniae* isolates was screened for *armA* and *rmtB* using primers (Alpha DNA,USA) that reported in Table 1. The lyophilized product of primers was dissolved in sterilized deionized distilled water (ddDW) (Promega,USA) to obtain 100 pmol/ $\mu$ l and then diluted to 10 pmol/ $\mu$ l according to the informations of manufacturing company. The Polymerase Chain Reaction (PCR) was performed by using Thermal Cycler (BioRad,USA) at a volume of 20 $\mu$ l which consists of 10 $\mu$ l Go Taq Green Master Mix (Promega,USA), 2 $\mu$ l template DNA, 1 $\mu$ l F-primer, 1 $\mu$ l R-primer, and 6 $\mu$ l ddDW (15). The PCR of *armA* and *rmtB* was performed under the optimal conditions that reported in Table 2 and 3, respectively (16).

**Table 1. Sequence of Primers used in this study.**

Genes	Primer Sequence (5'- 3')	Size (bp)	Reference
<i>armA</i>	F:CCGAAATGACAGTTCCTATC	846	(16)
	R:GAAAATGAGTGCCTTGGAGG		
<i>rmtB</i>	F:ATGAACATCAACGATGCCCTC	769	(16)
	R:CCTTCTGATTGGCTTATCCA		

**Table 2. The Optimal Conditions for amplifying *armA* by PCR.**

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	55	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

**Table 3. The Optimal Conditions for amplifying *rmtB* by PCR.**

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	60	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

**Gel Electrophoresis:**

The PCR products were run on 1% agarose gel that stained with 0.5 µg/ml ethidium bromide in 1X TAE buffer (Promega, USA) using DNA ladder (100-1500bp) supplied by Promega (USA), as a marker of DNA size. The electrophoresis was performed at 100 V for 80 min. The UV-Transilluminator (Major Science, Taiwan) was used for observation of PCR products under 320nm UV light (17).

**DNA Sequencing:**

The sequencing of 2 PCR products that referred to the *armA* was done by genetic analyzer (Macrogen Inc., South Korea). The result was compared with the reference database available in the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov>) using BioEdit program version 7.1 (DNASTAR, Madison, WI, USA) (<http://bioedit.software.informer.com/7.1/>) (18). The variations were translated into amino acid sequences using expasy online program (<http://web.expasy.org/translate/>) (19).

**Phylogenetic Tree Construction:**

The observed PCR amplicons variants of *armA* genetic loci were compared with the neighbor homologous sequences using NCBI-BLASTn suite ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch)). The blast results of the observed variants were aligned and constructed using Clustal Omega and Simple Phylogeny Tools, respectively (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). A full inclusive tree, including the observed variants was visualized as a polar cladogram using Figtree Tool (<http://tree.bio.ed.ac.uk/software/figtree/>).

**Results and Discussion:**

Fifty isolates were identified as *K. pneumoniae* (Table 4). The bacteria were found at high prevalence in urine 15 (30%) followed by

blood 12 (24%), whereas sputum and wounds were obtained 9 (18%) isolates and finally burns were obtained 5 (10%) isolates. Several studies revealed that the most common site of *K. pneumoniae* infections is UTIs followed by bloodstream infections, pneumonia, and burns and wounds infections (3,20,21). Other studies collected *K. pneumoniae* from other cases including, pus, stool, cerebrospinal fluid, and catheters (20,21,22,23,24).

**Table 4. Distribution of *Klebsiella pneumoniae* isolates.**

Samples	Isolates No.(%)
Urine	15(30)
Blood	12(24)
Sputum	9(18)
Wounds	9(18)
Burns	5(10)
Total	50(100)

The results of MICs exhibited that *K. pneumoniae* isolates showed higher resistance against third and fourth generation of cephalosporins than aminoglycosides and carbapenems, as listed in Table 5.

**Table 5. MICs Values of *Klebsiella pneumoniae* isolates.**

Antibiotic	Resistance Values of MICs (µg/ml)	Resistant Isolates No.(%)
Gentamicin	≥16	25(50)
Amikacin	≥64	22(44)
Ertapenem	≥8	21(42)
Imipenem	4- ≥16	18(36)
Ceftriaxone	4- ≥64	43(86)
Ceftazidime	16-64	42(84)
Cefepime	4- ≥16	40(80)

Regarding aminoglycosides, 25(50%) of *K. pneumoniae* isolates were resistant to gentamicin with MIC ≥16µg/ml, while 22(44%) of isolates

were resistant to amikacin with MIC  $\geq 64$   $\mu\text{g/ml}$ . The ability of *K. pneumoniae* to produce AMEs confers high resistance against aminoglycosides, in addition to overexpression of efflux pumps and loss of porins from cell membrane (5). The emergence of 16S rRNA methylases provides high resistance for all common aminoglycosides (7).

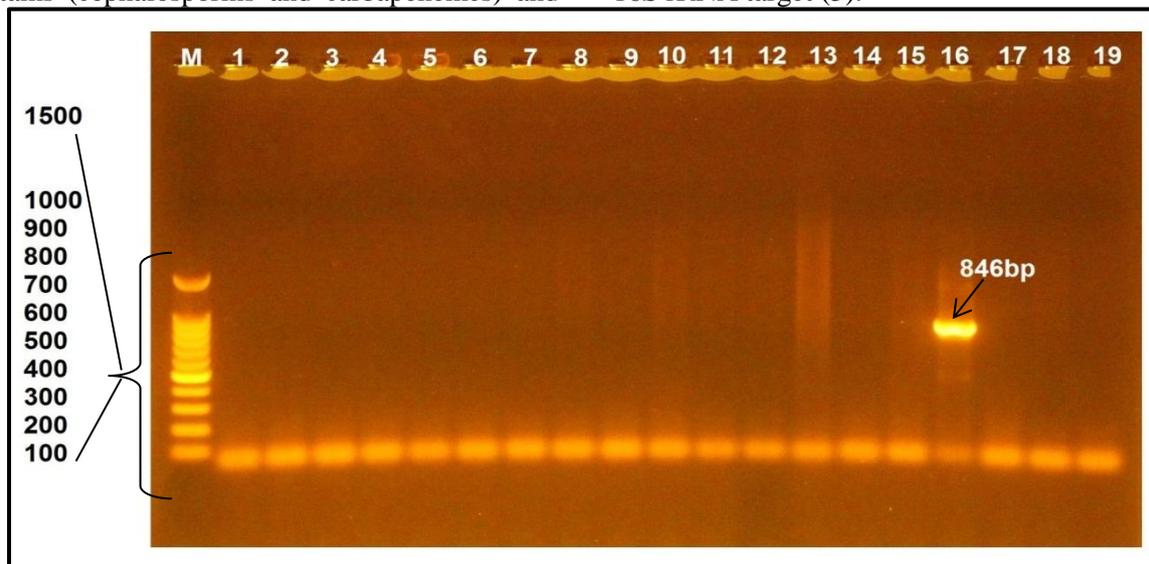
Concerning carbapenems, 21(42%) of *K. pneumoniae* isolates were resistant to ertapenem with MIC  $\geq 8$   $\mu\text{g/ml}$  and 18(36%) of isolates were resistant to imipenem with MIC 4-  $\geq 16\mu\text{g/ml}$ . The resistance of *K. pneumoniae* for carbapenems is due to the production of carbapenemases which confer resistance not only for carbapenemes but also for non  $\beta$ -lactams particularly aminoglycosides (25).

Concerning cephalosporins, the current study revealed that 43(86%) of *K. pneumoniae* isolates were resistant to ceftriaxone with MIC 4-  $\geq 64$   $\mu\text{g/ml}$ , 42(84%) of isolates were resistant to ceftazidime with MIC 16-64  $\mu\text{g/ml}$ , and 40(80%) of isolates were resistant to cefepime with MIC 4-  $\geq 16\mu\text{g/ml}$ . The production of ESBLs and AmpC-  $\beta$ -lactamases, in addition of carbapenemases is responsible for high resistance against third and fourth generation of cephalosporins (25).

The results revealed that 25(50%) of *K. pneumoniae* isolates exhibited co-resistant for both  $\beta$ -lactams (cephalosporins and carbapenemes) and

aminoglycosides (gentamicin and amikacin). The studies (26,27,28) illustrated that the dissemination of 16S rRNA methylases encoding genes among *K. pneumoniae* is mediated by conjugative plasmids which confer resistance for both aminoglycosides and  $\beta$ -lactams. Moreover, 25(50%) of *K. pneumoniae* isolates were positive for ESBLs. The production of ESBLs becomes a serious threat because the rapid dissemination of ESBLs encoding plasmids among the strains of *K. pneumoniae* by HGT (29). The problem becomes more complicated because ESBLs confer resistance against most common clinically used third and fourth generation of cephalosporins and even against aminoglycosides and quinolones (29,30). World Health Organization (WHO) reported that the endemic rates of ESBLs producing *K. pneumoniae* reached 50% throughout the world (31).

The *armA* with 846bp was found in 5(10%) of *K. pneumoniae* isolates (Fig.1). Moreover, *rmtB* was not found among *K. pneumoniae* isolates. Although the low prevalence of these genes, they can easily spread among *K. pneumoniae* isolates and even among other species by HGT because they are mainly carried on plasmids (27). The *armA* and *rmtB* encode for 16S rRNA methylases which confer high resistance against all aminoglycosides by preventing these drugs from binding with their 16S rRNA target (5).



**Figure 1. Gel Electrophoresis of PCR products showed *armA* gene with 846bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500bp). Lane 16 represents DNA from *K. pneumoniae* isolate.**

DNA sequencing of *armA* exhibited the occurrence of two point mutations of transition which led to the replacement of guanine with adenine (G>A) at the position 1424707 (Fig.2). The translation of protein revealed that these point mutations were missense mutations by which serine

was replaced with phenylalanine (Fig.3). These mutations may have elevated MICs values of both gentamicin and amikacin. The mutant DNA sequences were registered in NCBI at the link <https://www.ncbi.nlm.nih.gov/nuccore/LC373258>.

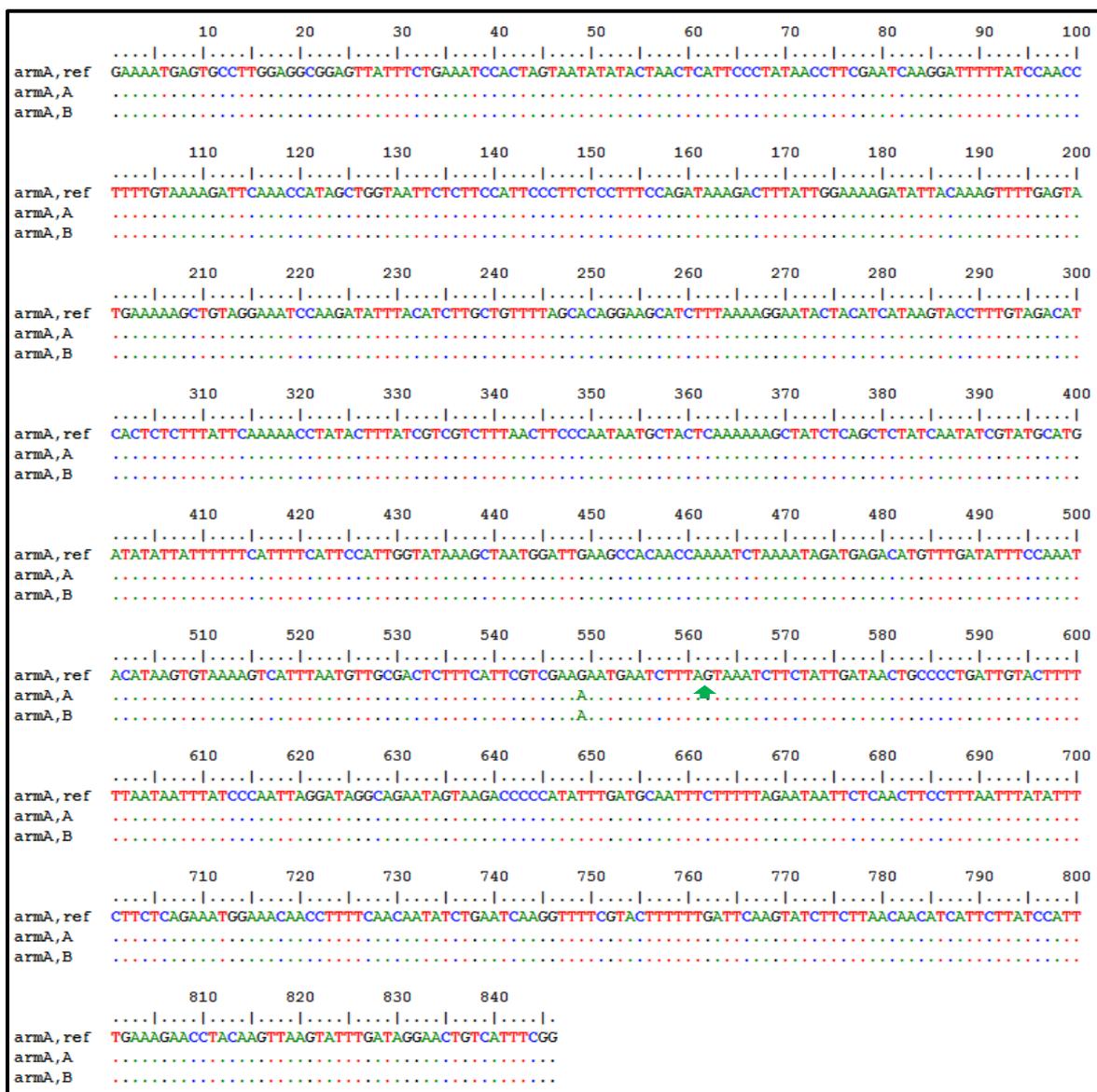


Figure 2. DNA Sequences Alignment of the observed native strains with their corresponding reference sequences of the 846bp amplicon of *armA* gene. Each substitution SNP was highlighted according to its position in the PCR products.

MDKNDVVKILESCKYENLDS DIVEKVV SISEKKYKLKEVENYSK KKLHQIWGSYYSAYPNWDKLLKKYNQ  
 GQLSIEDLLKIHSS TNERVATLND FYTYVFGNIKHVSSILDFGCGFNPLALYQWNENEKIIYHAYDIDRAEIAFLS  
 SIIGKLLTKIKYRFLNKESDVYKGT YDVFLLKMLPVLKQQDVNILD FLQLFHTQNFVISFPIKSLSGKEKGMEE  
 NYQLWFESFTKGWIKILDSKVIGNELVYITSGFQK

**A: Referring amino acids sequences**

MDKNDVVKILESCKYENLDS DIVEKVV SISEKKYKLKEVENYSK KKLHQIWGSYYSAYPNWDKLLKKYNQ  
 GQLSIEDLLKIHFS TNERVATLND FYTYVFGNIKHVSSILDFGCGFNPLALYQWNENEKIIYHAYDIDRAEIAFLS  
 SIIGKLLTKIKYRFLNKESDVYKGT YDVFLLKMLPVLKQQDVNILD FLQLFHTQNFVISFPIKSLSGKEKGMEE  
 NYQLWFESFTKGWIKILDSKVIGNELVYITSGFQK

**B: Mutant amino acids sequences**

Figure 3. Sequence of referring amino acids (A) and mutant amino acids (B), resulting from the translation of mutant DNA of *armA*. S:serine;F:phenylalanine.

Phylogenetic tree (Fig.4) revealed that both *armA* variants that shared the same mutation (G>A) occupied a distinctive position within this tree. Despite the distinctive characterization of the *armA* variants, a slight deviation from *K. pneumoniae*

species was noticed concerning these variants. This fact is obviously observed in the current constructed comprehensive phylogenetic tree as both variants were positioned between *K. pneumoniae* and *E.coli* deposited referring sequences. The observed *armA*

variants were precisely positioned near *E. coli* (acc. no. LC056143.1) and *K. pneumoniae* (acc. no. CP021960.1, CP012997.1, CP020841.1), respectively. Though this *armA* based tree was highly occupied by *Acinetobacter baumannii* species as this organism occupied about two portions of this tree. This graphical representation of *armA* based tree indicated a better ability of this 846 bp genetic fragment to successfully identify the current local *K. pneumoniae* isolates. This ability came from the reports that found such genetic fragment as one of the most commonly identified 16S rRNA methylases encoding genes in *K.*

*pneumoniae* (27). Moreover, the limited number of *K. pneumoniae* that found in this tree may sustain this idea. This observation provided an additional inclusive indication about the identification of these local studied isolates.

In conclusion, the selective pressure of antibiotics had led to emergence of co-resistance among *K. pneumoniae* isolates for both  $\beta$ -lactams and aminoglycosides. 16S rRNA methylases encoding genes were observed at low prevalence among *K. pneumoniae* isolates but confer resistance against almost all common clinically used aminoglycosides.

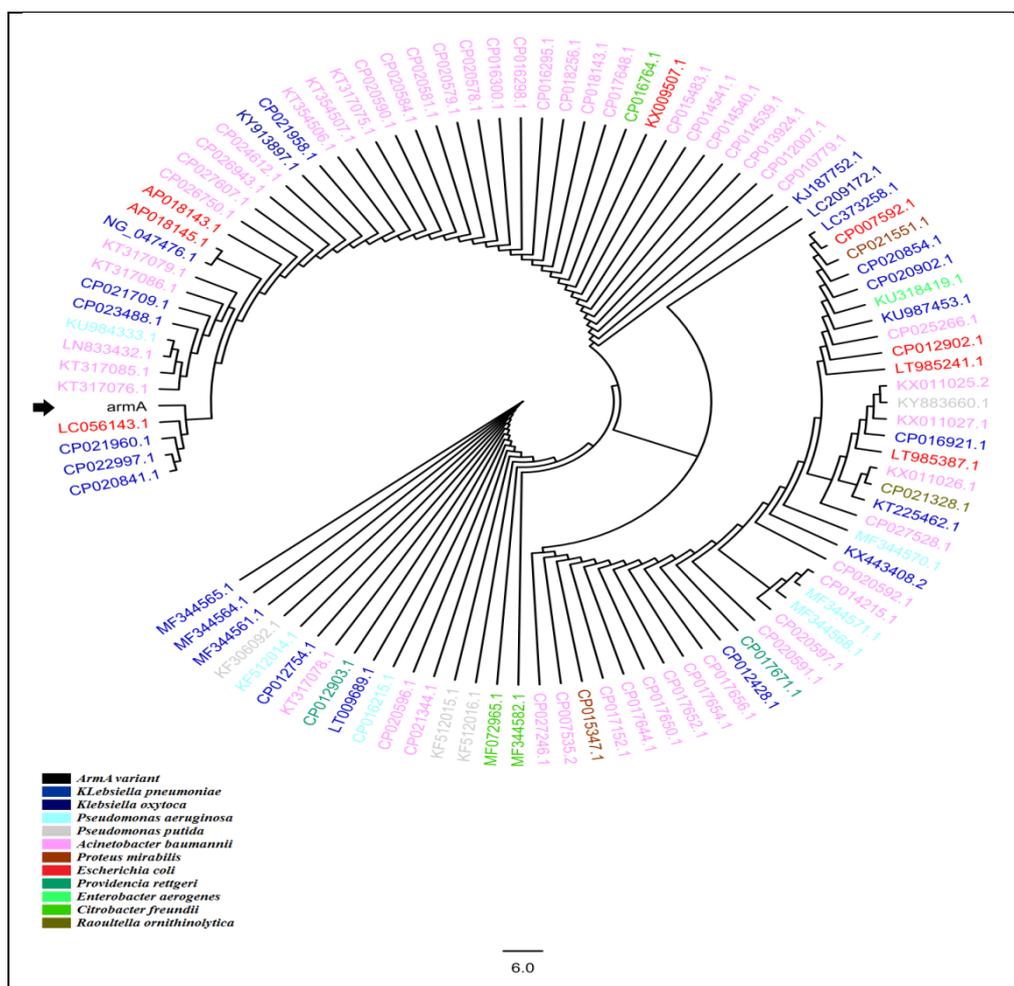


Figure 4. Comprehensive Phylogenetic tree of the 846bp genetic fragment of *K. pneumoniae* local isolates. The black color refers to the sequenced variants, while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to Genbank accession numbers of each referring species. The number “6.0” at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

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**Conflicts of Interest: None.**

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## تشخيص انزيمات مثيلة 16S rRNA و المقاومة المشتركة مع البيبتالاكتام في عزلات *Klebsiella pneumoniae* من المرضى العراقيين

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### الخلاصة:

تم الحصول على 50 عزلة من بكتريا *Klebsiella pneumoniae* من 150 عينة سريرية، اذ شخصت حسب الصفات المظهرية والكيموحيوية. جمعت العزلات من حالات سريرية مختلفة شملت الادراج 15 (30%) و الدم 12 (24%) و القشع 9 (18%) و الجروح 9 (18%) و الحروق 5 (10%). اظهرت نتائج التراكيز المثبطة الدنيا MICs ان 25 (50%) عزلة كانت مقاومة لمضاد gentamicin ( $\leq 16$  مايكروغرام/مل) و 22 (44%) عزلة مقاومة لمضاد amikacin ( $\leq 64$  مايكروغرام/مل) و 21 (42%) عزلة مقاومة لمضاد ertapenem ( $\leq 8$  مايكروغرام/مل) و 18 (36%) عزلة مقاومة لمضاد imipenem (4- $\geq 16$  مايكروغرام/مل) و 43 (86%) عزلة مقاومة لمضاد ceftriaxone (4- $\leq 64$  مايكروغرام/مل) و 42 (84%) عزلة مقاومة لمضاد ceftazidime (16-64 مايكروغرام/مل) و 40 (80%) عزلة مقاومة لمضاد cefepime (4- $\leq 16$  مايكروغرام/مل). تم تشخيص المقاومة المشتركة لكل من مضادات البيبتالاكتام و الامينوكلايكوسيدات ضمن 25 (50%) من عزلات بكتريا *K. pneumoniae*. تم تشخيص انزيمات البيبتالاكتامز واسعة الطيف ESBLs ضمن 25 (50%) من عزلات بكتريا *K. pneumoniae*. اظهرت نتائج التحري عن الجينات المشفرة لانزيمات مثيلة 16S rRNA ان الجين *armA* كان موجودا في 5 (10%) من عزلات بكتريا *K. pneumoniae*. من جهة اخرى اظهرت النتائج عدم وجود الجين *rmtB* في عزلات بكتريا *K. pneumoniae*. اظهر تحليل تتابع الحامض النووي DNA للجين *armA* وجود طفرات انحشار اثرت في ترجمة البروتين من خلال استبدال الاحماض الامينية بحيث ادى ذلك الى زيادة في قيم التراكيز المثبطة الدنيا MICs لكل من المضادين gentamicin و amikacin. تم تسجيل هذه التغيرات في موقع NCBI تحت الرقم التسلسلي LC373258. ان الشجرة التطورية لتغيرات الجين *armA* اظهرت ان هناك انحراف طفيف لهذه التغيرات عن النوع *K. pneumoniae*.

الكلمات المفتاحية: انزيمات البيبتالاكتامز واسعة الطيف ESBLs ، *Klebsiella pneumoniae* ، الشجرة التطورية ، انزيمات مثيلة 16S rRNA.