



ISSN: 0067-2904

Investigation for some Aminoglycosides Modifying Enzymes- Encoding Genes and Co-Resistance to Fluoroquinolones among *Klebsiella pneumoniae* Isolates from Different Clinical Cases

Mustafa Suhel Mustafa*, Rana Mujahid Abdullah

Department of Biology, College of Education for Pure Science/Ibn Al-Haitham, University of Baghdad, Baghdad, Iraq

Received: 31/3/2020

Accepted: 7/5/2020

Abstract

In this study, we investigated the prevalence of aminoglycosides modifying enzymes (AMEs)-encoding genes, including *aac(3')-II*, *ant(3')-I*, *aph(3')-VI*, and *aac(6')-Ib-cr* and their potential effect on the development of resistance to aminoglycosides and fluoroquinolones in clinical isolates of *Klebsiella pneumoniae*. According to the phenotypic and biochemical characteristics of 150 clinical samples, 50 (33%) isolates were identified as *K. pneumoniae*. These isolates were collected from different clinical sources, including urine (15, 30%), blood (12, 24%), sputum (9, 18%), wounds (9, 18%), and burns (5, 10%). The minimum inhibitory concentrations (MICs) assay revealed that the resistance values of isolates were 25 (50%) to gentamicin ($\geq 16 \mu\text{g/ml}$), 21 (42%) to amikacin ($\geq 64 \mu\text{g/ml}$), 15 (30%) to ciprofloxacin ($\geq 4 \mu\text{g/ml}$), and 11 (22%) to levofloxacin ($\geq 8 \mu\text{g/ml}$). Genotypic detection revealed that *aac(3')-II*, *aac(6')-Ib-cr*, *aph(3')-VI*, and *ant(3')-I* were found in 47 (94%), 38 (76%), 18 (36%), and 8 (16%) of *K. pneumoniae* isolates, respectively. The co-resistance pattern for both aminoglycosides and fluoroquinolones was detected in 14 (28%) isolates, of these 10 (71.4%) harbored *aac(6')-Ib-cr*. DNA sequencing for some isolates revealed the presence of point and frameshift mutations in the studied genes. Our study findings suggest that the presence of missense and frameshift mutations may contribute to the elevated resistance to amikacin and gentamicin. The increased prevalence of AMEs-encoding genes among *K. pneumoniae* isolates could contribute in reducing susceptibility to amikacin and gentamicin. The co-resistance pattern for aminoglycosides and fluoroquinolones was highly associated with the presence of the *aac(6')-Ib-cr* gene.

Keywords: AMEs, Aminoglycosides, Fluoroquinolones, *Klebsiella pneumoniae*, Co-resistance.

التحري عن بعض الجينات المشفرة للأنزيمات المحورة للأمينوكلايكوسيدات و المقاومة المشتركة مع مضادات الفلوروكينولونات في عزلات لبكتيريا *Klebsiella pneumoniae* من حالات سريرية مختلفة

مصطفى سهيل مصطفى*، رنا مجاهد عبدالله

قسم علوم الحياة، كلية التربية للعلوم الصرفة/ابن الهيثم، جامعة بغداد، بغداد، العراق

*Email: mustavasuhel@gmail.com

الخلاصة

هدفت هذه الدراسة الى التحري عن تواجد الجينات المشفرة للأنزيمات المحورة للامينوكلايكوسيدات *aac(3)-II* و *ant(3)-I* و *aph(3)-VI* و *aac(6)-Ib-cr* و تأثيرها الفعال في تطور المقاومة لمضادات الامينوكلايكوسيدات و الفلوروكينولونات في عزلات سريرية لبكتيريا *Klebsiella pneumoniae*. تم الحصول على 50 (33%) عزلة من بكتيريا *K. pneumoniae* من 150 عينة سريرية , اذ شخّصت حسب الصفات المظهرية و الكيموحيوية. جمعت العزلات من حالات مختلفة شملت الادرار 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 قيم المقاومة للعزلات كانت 25 (50%) لمضاد gentamicin (≤ 16) مايكروغرام(مل) و 21 (42%) لمضاد amikacin (≤ 64) مايكروغرام(مل) و 15 (30%) لمضاد ciprofloxacin (≤ 4) مايكروغرام(مل) و 11 (22%) لمضاد levofloxacin (≤ 8) مايكروغرام(مل). اظهرت نتائج التشخيص الجيني ان الجينات *aac(3)-II* و *aac(6)-Ib-cr* و *aph(3)-VI* و *ant(3)-I* كانت موجودة ضمن 47 (94%) و 38 (76%) و 18 (36%) و 8 (16%) من عزلات بكتيريا *K. pneumoniae* على التوالي. تم تشخيص نمط المقاومة المشتركة لمضادات الامينوكلايكوسيدات و الفلوروكينولونات في 14 (28%) و من بينها 10 (71.4%) امتلكت الجين *aac(6)-Ib-cr*. اظهر تحليل تتابع الحامض النووي DNA لبعض من العزلات وجود طفرات نقطية و طفرة frameshift في الجينات المدروسة. ان طفرة الانحشار و طفرة frameshift من الممكن ان تكون قد ادت الى زيادة في قيم المقاومة للتراكيز المثبطة الدنيا MICs لمضاد gentamicin و amikacin. ان الانتشار المتزايد للجينات المشفرة للأنزيمات المحورة للامينوكلايكوسيدات في عزلات بكتيريا *K. pneumoniae* قد ساهم في اختزال حساسيتها لمضاد gentamicin و amikacin. ان نمط المقاومة المشتركة لمضادات الامينوكلايكوسيدات و الفلوروكينولونات كان مرتبطا بشكل واضح بتواجد الجين *aac(6)-Ib-cr*.

Introduction

Klebsiella pneumoniae is a Gram-negative bacillus which is classified as a member of the family Enterobacteriaceae, with cells that are usually surrounded by a polysaccharide capsule that interferes with the host defense by inhibiting phagocytosis [1]. Once humans acquire *K. pneumoniae* from environment or hospital, it can colonize the mucosal membranes of the nasopharynx and gastrointestinal tract [2]. This colonization can be progressed into serious infections, especially in hospitalized immune-compromised individuals, making them more susceptible to develop urinary tract infections (UTIs), bloodstream infections, pneumonia, surgical and burn infections, meningitis, cellulitis, and liver abscess [2].

Throughout the last decade, *K. pneumoniae* represented a major worldwide burden concerning antibiotics resistance. As a result of the selective pressure of the antibiotics, *K. pneumoniae* continuously undergoes mutations and acquires antibiotic resistance genes by horizontal gene transfer [3]. This enables *K. pneumoniae* to evolve multiple resistance mechanisms against most of the common antibiotics in clinical use. Such effect could lead to the emergence of the multidrug resistant (MDR) and extremely drug resistant (XDR) strains that pose a high risk to public health and are often associated with prolonged hospitalization along with high rates of morbidity and mortality [3].

Aminoglycosides and fluoroquinolones become among the last antimicrobial options used to treat infections with *K. pneumoniae*. Aminoglycosides 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 the blockage of protein synthesis [4]. On the other hand, fluoroquinolones act by preventing the synthesis and replication of the bacterial DNA by targeting topoisomerases (DNA gyrase and topoisomerase IV) [5].

The aminoglycosides modifying enzymes (AMEs) are the most common mechanism employed by *K. pneumoniae* to interfere with the action of aminoglycosides [6]. Three families of the AMEs were detected in *K. pneumoniae*, including aminoglycosides acetyltransferases (AACs), aminoglycosides phosphotransferases (APHs), and aminoglycosides nucleotidyltransferases (ANTs). These are known to inhibit the active role of aminoglycosides by N-acetylation, O-phosphorylation, and O-nucleotidylation of the aforementioned drugs, respectively [4,6]. Interestingly, the frequency of

mutations in the AMEs-encoding genes increases the diversity of these genes through the contribution in the emergence of new alleles. This results in reducing the active role of common used aminoglycosides which imposes a great health challenge for both patients and physicians that can be inevitably difficult to be confronted and controlled [4,6].

aac(6')-Ib-cr is an allele of the *aac(6')-Ib* gene that belongs to the AACs family and confers co-resistance for both aminoglycosides and fluoroquinolones [7]. Initially, it was detected on the plasmid in *K. pneumoniae* as one of the plasmid mediated quinolone resistance (PMQR) mechanisms, but recently was found on the chromosome of this species [5]. *aac(6')-Ib-cr* deactivates fluoroquinolones that have amino nitrogen on the piperazinyl ring, the substrate of the encoded enzyme AAC(6')-Ib-cr. This enzyme is distinguished from the other AAC(6')-Ib enzymes by the presence of two unique amino acid substitutions, Trp102Arg and Asp179Try, that are required for acetylating the fluoroquinolones [5,7].

This study was conducted to investigate the prevalence of some AMEs-encoding genes, namely *aac(3')-II*, *ant(3'')-I*, *aph(3')-VI*, and *aac(6')-Ib-cr*, along with their effects on the resistance to aminoglycosides and fluoroquinolones among clinical isolates of *K. pneumoniae*.

Materials and Methods

Sampling

One hundred fifty clinical samples were recovered from patients at different hospitals in Baghdad during the period between July 2018 to October 2018. The samples were collected from different specimen types, including urine, blood, sputum, wounds, and burns. All urine and sputum samples were collected in sterile disposable containers (Himedia, India), while blood samples were collected in EDTA tubes (Apex Bio Medicals, India). Also, sterile transport swabs (Pal Surgical Works, India) were used for collecting the samples from wound and burn infections. The instructions of the Ethics Committee of the Iraqi Ministry of Health were followed to conduct this study according to the official ethical approval numbered 31864.

Laboratory Diagnosis

Initially, the clinical samples were cultured on Gram negative selective media, including MacConkey agar and Eosin Methylene Blue agar (Oxoid, UK), at incubation conditions of 37°C for 24 hrs [8]. Then, lactose fermenting colonies were identified by performing several conventional biochemical tests, including oxidase, catalase, urease, and IMVIC tests [8]. Ultimately, the Vitek 2 Compact System (BioMerieux, France) was used to confirm the identified bacteria [9].

Susceptibility Test

The susceptibility test of all investigated *K. pneumoniae* isolates was done by determining minimum inhibitory concentrations (MICs) using Vitek 2 AST-GN cards (BioMerieux, France) [9]. The antimicrobial susceptibility was assayed to Gentamicin (4, 16, 32 µg/ml), Amikacin (8, 16, 64 µg/ml), Ciprofloxacin (0.5, 2, 4 µg/ml), and Levofloxacin (0.25, 0.5, 2, 8 µg/ml). Several pure colonies from an overnight culture of *K. pneumoniae* were transferred to 3 ml normal saline to form a homogenous bacterial suspension with a turbidity range of 0.50-0.63. Then, 145 µl of the suspension was transferred into 3 ml normal saline in a second tube which was placed in a cassette opposite the AST-GN card [9]. The results were interpreted following CLSI [10].

DNA Extraction

DNA was extracted from bacterial isolates using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the information of the manufacturing company. Briefly, 1400 µl of overnight *K. pneumoniae* broth culture was centrifuged (ThermoScientific, USA) for 2 min at 1300 rpm, then the cell pellet was collected in an Eppendorf tube (Promega, USA). The subsequent steps of DNA extraction included the addition of nuclei lysis solution, RNase solution, and protein precipitation solution. After that, DNA was precipitated by adding isopropanol and 70% ethanol. Finally, DNA pellet was rehydrated by adding rehydration solution and kept on ice at -20°C. The concentrations and purity of the extracted DNA were measured by Nanodrop spectrophotometer (BioDrop, UK) [11].

Molecular Screening

The extracted DNA was used as a template to detect the presence of *aac(3')-II*, *ant(3'')-I*, *aph(3')-VI*, and *aac(6')-Ib-cr*, utilizing Polymerase Chain Reaction (PCR). The lyophilized primers (Alpha DNA, USA, reported in Table-1) were dissolved in sterilized deionized distilled water (ddDW) (Promega, USA) to obtain 100 pmol/µl and then diluted to 10 pmol/µl according to the instructions of

the manufacturing company. The PCR mixture was prepared at a volume of 20 μ l, which contained 10 μ l Go Taq Green Master Mix (Promega, USA), 2 μ l template DNA, 1 μ l of both forward and reverse primers, and 6 μ l ddDW [11]. The amplification was achieved using Thermal Cycler (BioRad, USA) and started by initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 5 min, while it was finalized with a final extension at 72°C for 5 min [12].

Table 1-Sequence of primers used in the present study

Genes	Primer Sequence (5' - 3')	Size (bp)	Reference
<i>aac(3')-II</i>	F: ATATCGCGATGCATACGCGG R: GACGGCCTCTAACCGGAAGG	877	12
<i>ant(3'')-I</i>	F: CATCATGAGGGAAGCGGTG R: GACTACCTTGGTGATCTCG	787	12
<i>aph(3')-VI</i>	F: ATGGAATTGCCCAATATTATT R: TCAATTCAATTCATCAAGTTT	780	12
<i>aac(6')-Ib-cr</i>	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	482	12

aac: aminoglycoside acetyltransferase, *ant*: aminoglycoside nucleotidyltransferase, *aph*: aminoglycoside phosphotransferase, *aac(6')-Ib-cr*: aminoglycoside 6'-N-acetyltransferase-ciprofloxacin variant, F: forward, R: reverse, bp: base pair.

All PCR products were loaded into wells of 1% agarose gel and stained with 0.5 μ g/ml ethidium bromide in 1X TAE buffer (Promega, USA) at 100 V for 80 min. The DNA ladder (100-1500 bp) (Promega, USA) was used as a marker for the molecular weight of DNA bands. The DNA bands were photographed under 320 nm UV light using UV-Transilluminator (Major Science, Taiwan) [11].

DNA Sequencing

Two *K. pneumoniae* PCR products from each of *aac(3')-II*, *ant(3'')-I*, and *aph(3')-VI* were sent to Macrogen company in South Korea for sequencing the DNA fragments from both directions using the Genetic Analyzer (Macrogen Inc., South Korea). These isolates were selected for sequencing due to show resistance to the aminoglycosides used in this study. Consequently, the potential mutations in these isolates may be associated with the high amikacin and/or gentamicin resistance rates. Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotations and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local isolates with the most homologous reference DNA sequences of *K. pneumoniae* retrieved from the National Center for Biotechnology Information (NCBI), the virtual positions and other details of the studied PCR fragments were identified using the NCBI BLASTn program (<https://www.ncbi.nlm.nih.gov>) and BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA) (<http://bioedit.software.informer.com/7.1/>), respectively. The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

Regarding protein translation, the amino acid sequences of the targeted proteins were also retrieved from NCBI. The observed variants in the coding portions were translated into a reading frame, that were 3'-5' frame3 for *aac(3')-II* and 5'-3' frame1 for *ant(3'')-I* and *aph(3')-VI*, corresponding to the referring amino acid residues, using the Expasy online program (<http://web.expasy.org/translate/>).

Results and Discussion

In view of the results obtained from biochemical examinations and Vitek 2 Compact System analysis, 50 isolates were identified as *K. pneumoniae*, recovered from urine 15 (30%), blood 12 (24%), sputum 9 (18%), wounds 9 (18%), and burns 5 (10%). Overall, the most common site of *K. pneumoniae* infection was the UTIs, followed by bloodstream infections. Furthermore, approximately 13% of wound infections were caused by *K. pneumoniae* [13]. *K. pneumoniae* can also be collected from other body sites, including pus, stool, cerebrospinal fluid, and catheters [2,13,14,15]. At each of these sites, *K. pneumoniae* represents an opportunistic pathogen and endemic infections can be progressed [2].

The results of MICs, listed in Table-2, exhibited that 25 (50%) of *K. pneumoniae* isolates were resistant to gentamicin, with MIC \geq 16 μ g/ml, while 21 (42%) isolates were resistant to amikacin, with

MIC ≥ 64 $\mu\text{g/ml}$. The resistance rates reported by Zafer and colleagues [16] were nearly in line with those observed by this study for both antibacterial agents. In contrast, the investigation of Mokhtari *et al.* [17] reported lower resistance rates to gentamicin and amikacin. The ability of *K. pneumoniae* to produce a wide variety of AMEs confers high resistance against aminoglycosides, in addition to overexpression of efflux pumps and loss of porins from cell membrane [4,6]. The emergence of 16S rRNA methylases provides high resistance to all common aminoglycosides [4].

Table 2-MICs Values of *Klebsiella pneumoniae* isolates

Antibiotic	Resistant Values of MICs ($\mu\text{g/ml}$)	Resistant Isolates No.(%)
Gentamicin	≥ 16	25 (50)
Amikacin	≥ 64	21 (42)
Ciprofloxacin	≥ 4	15 (30)
Levofloxacin	≥ 8	11 (22)

In respect to fluoroquinolones, 15 (30%) of *K. pneumoniae* isolates were resistant to ciprofloxacin (MIC ≥ 4 $\mu\text{g/ml}$), while 11 (22%) isolates were resistant to levofloxacin (MIC ≥ 8 $\mu\text{g/ml}$). Higher ciprofloxacin and levofloxacin resistance values were demonstrated by previous findings [16,18]. It was found that the resistance for ciprofloxacin and/or levofloxacin among *K. pneumoniae* isolates was correlated with the high prevalence of *aac(6')-Ib-cr* in these isolates. Alongside, there are other mechanisms that contribute to fluoroquinolones resistance. These include the accumulation of chromosomal mutations in the topoisomerases encoding genes (*gyrA* and *parC*), acquisition of other PMQR (*qnr* and *oqxAB*) that become widely spread even in other genera of Gram negative bacteria, particularly these involved in severe nosocomial infections [19], and the alternations of cell permeability through the loss of porins and overexpression of the MDR efflux pumps [5,7].

Genotypic screening revealed that *aac(3')-II* and *aac(6')-Ib-cr* genes were found at high prevalence, in which 47 (94%) and 38 (76%) of *K. pneumoniae* isolates possessed these genes with PCR products of 877 bp and 482 bp, respectively, as shown in Figures-(1 and 2). Previous studies [20,21] detected *aac(3')-II* at lower rates among their bacterial isolates as compared to this study. With regard to *aac(6')-Ib-cr*, the results obtained by previous reports [21,22,23] were relatively in line with that gained by this study, while lower rates were seen by others [24,25]. *aac(3')-II* encodes for aminoglycoside 3'-N-acetyltransferase type II [4]. This enzyme confers resistance against aminoglycosides by catalyzing the transfer of acetyl group from acetyl-CoA to aminoglycoside molecules, resulting in the loss of their antimicrobial activity [6]. On the other hand, *aac(6')-Ib-cr* gene encodes for aminoglycoside 6'-N-acyltransferase type Ib ciprofloxacin variant that can acetylate aminoglycosides and fluoroquinolones (bifunctional enzyme), leading to the reduction of susceptibility to these agents [5,7].

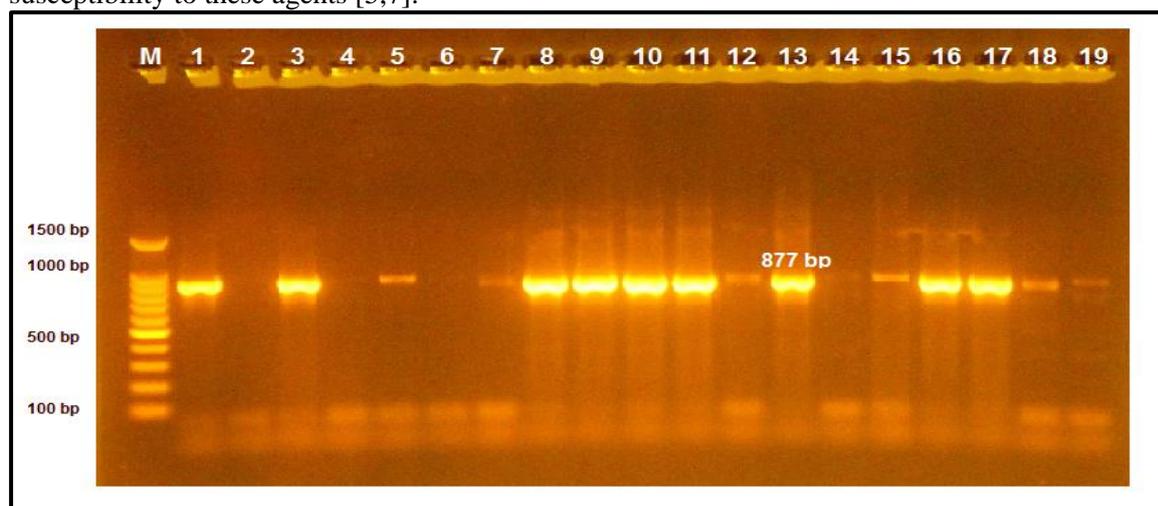


Figure 1- Gel electrophoresis of PCR products showing *aac(3')-II* gene with 877 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 1,3,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19 represent *Klebsiella pneumoniae* PCR positive isolates.

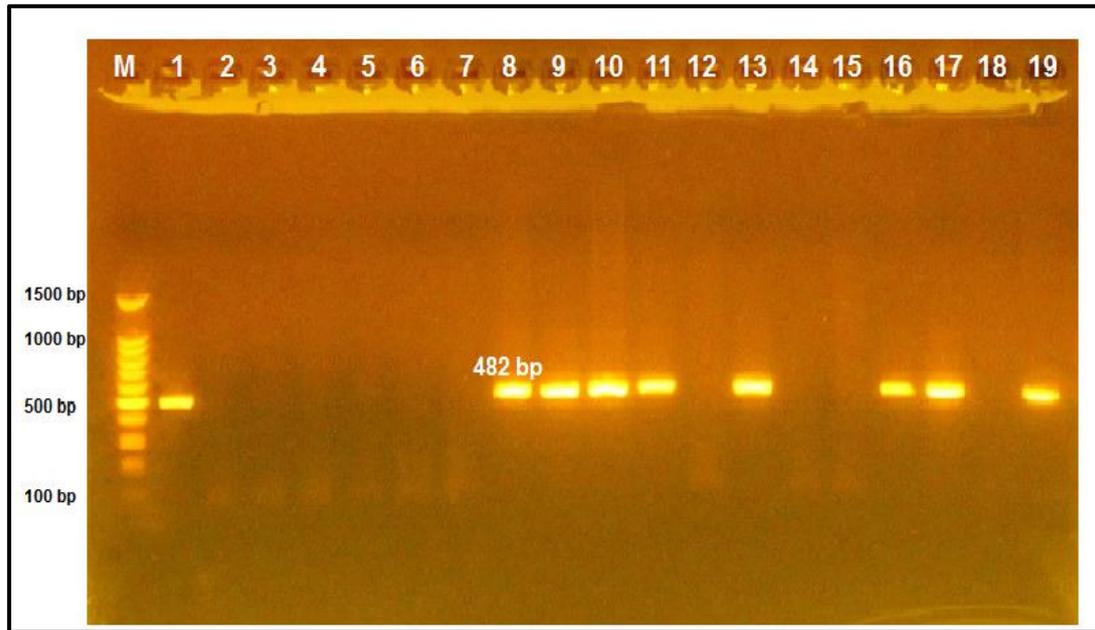


Figure 2- Gel electrophoresis showing PCR products of *aac(6)-Ib-cr* gene with 482 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 1,8,9,10,11,13,16,17,19 represent *Klebsiella pneumoniae* PCR positive isolates.

aph(3')-VI gene was observed in 18 (36%) of *K. pneumoniae* isolates with a PCR product of 780 bp (Figure-3). This rate of prevalence was lower than that observed by previously published articles [20,22]. *aph(3')-VI* encodes for aminoglycoside 3'-O-phosphotransferases type VI, which confers resistance to aminoglycosides by catalyzing the transfer of the γ -phosphoryl group from ATP to aminoglycoside molecules, causing a lack of their effectiveness against *K. pneumoniae* [4].

Furthermore, *ant(3'')-I* was identified in 8 (16%) of *K. pneumoniae* isolates with a PCR product size of 787 bp (Figure-4). However, earlier studies reported this gene at higher rates among their *K. pneumoniae* isolates [20,23]. *ant(3'')-I* encodes for aminoglycoside 3''-O-nucleotidyltransferases type I, which confers resistance to aminoglycosides by catalyzing the transfer of AMP from MgATP to aminoglycoside molecules, leading to the loss of their activity against *K. pneumoniae* [4].

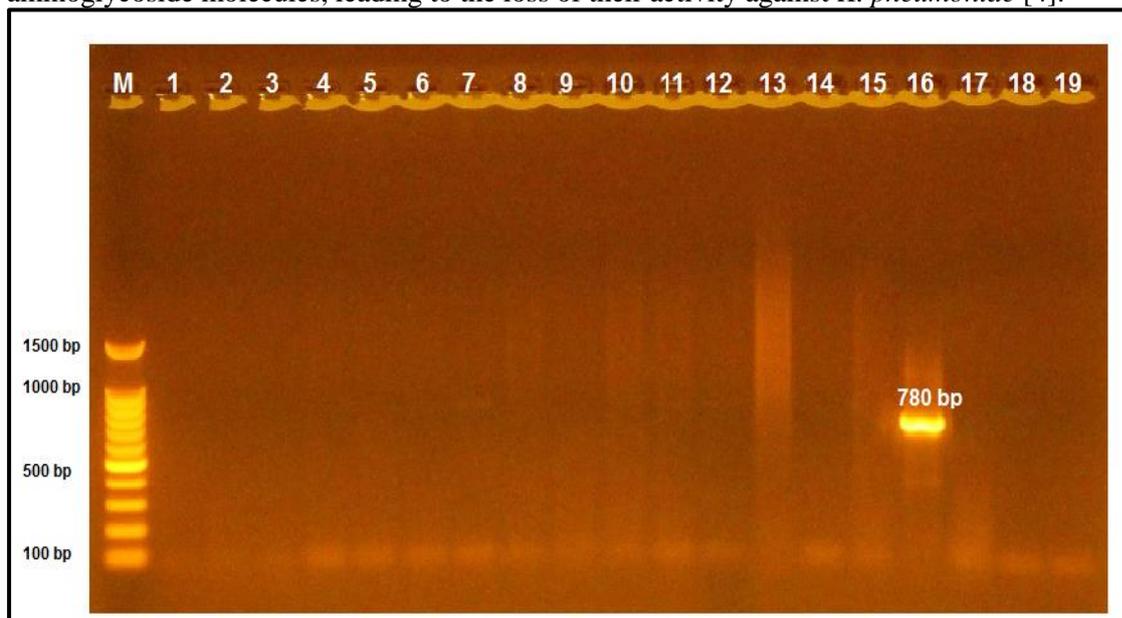


Figure 3- Gel electrophoresis of PCR products showing *aph(3')-VI* gene with 780 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lane 16 represents *Klebsiella pneumoniae* PCR positive isolate.

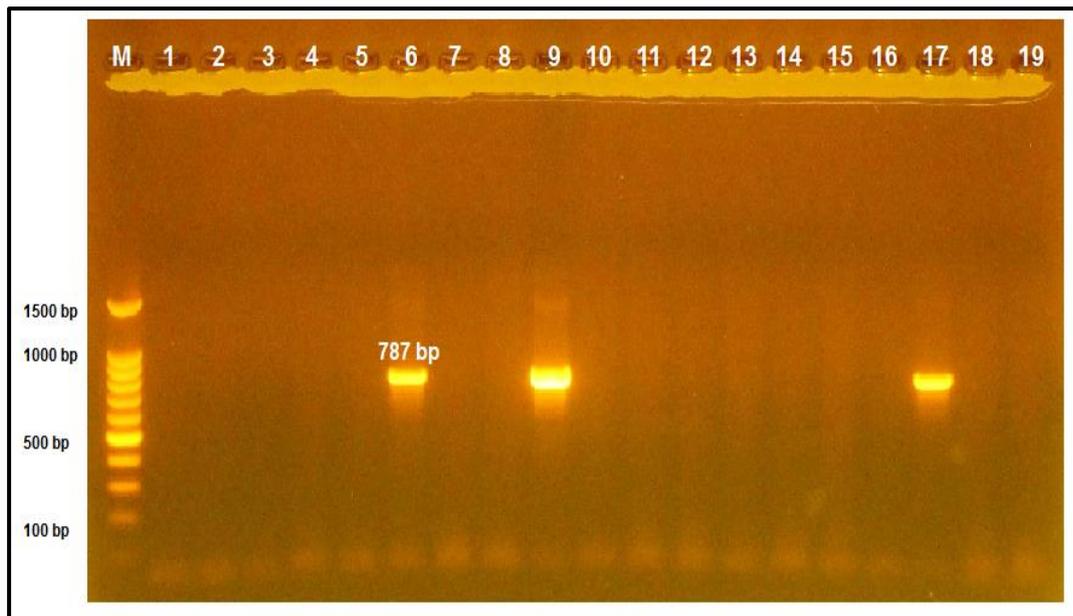


Figure 4- Gel electrophoresis of PCR products showing *ant(3'')-I* gene with 787 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 6,9,17 represent *Klebsiella pneumoniae* PCR positive isolates.

The difference in the prevalence rates of AMEs-encoding genes among the present study and the earlier reports can be attributed to the number and source of the studied isolates, working conditions in the laboratory, silent colonization by *K. pneumoniae* carrying AMEs-encoding genes, long term health care, misuse of antibiotics, and contact with the possible reservoir animals [26]. More serious consequences can arise from the fact that all these factors may facilitate the horizontal gene transfer of AMEs-encoding genes between high risk resistant strains of *K. pneumoniae* and normal flora of these bacteria that colonize gastrointestinal tract of healthy humans [3]

The results of the association between phenotype (MICs) and genotype (PCR) of aminoglycosides resistance, listed in Table-3, exhibited that 25 (50%) of *K. pneumoniae* isolates were resistant for amikacin and/or gentamicin, of these 23 (88%) isolates harbored one or more AMEs-encoding genes. Alongside, the phenotype (MICs)/ genotype (PCR) association value for fluoroquinolones resistance was 17 (34%) of *K. pneumoniae* isolates that showed resistance to ciprofloxacin and/or levofloxacin, among which 13 (76.4%) possessed *aac(6')-Ib-cr* genes. The co-resistance pattern for both aminoglycosides and fluoroquinolones was detected in 14 (28%) isolates, among which 10 (71.4%) harbored *aac(6')-Ib-cr*. As with other PMQR, *aac(6')-Ib-cr* confers low or moderate level resistance against fluoroquinolones, but it enhances the occurrence of chromosomal mutations in the topoisomerases encoding genes (*gyrA* and *parC*), resulting in high level fluoroquinolones resistance [5,7]. The co-resistance pattern plays an influential role in the continuous emergence of epidemic clones that employ various modes of resistance. Thus, pandemic clones can be evolved that constitute a substantial challenge for healthcare due to limited options of antimicrobial treatment [3].

Table 3- Association between Phenotype and Genotype of Aminoglycosides and Fluoroquinolones Resistance and Co-Resistance Patterns for both Agents in *K. pneumoniae* isolates

Isolate No.	Phenotypic Profile	Genotypic Profile	Co-Resistance Pattern
KP1	GEN, AMK	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP2	GEN, AMK, CIP	-	-
KP3	GEN, AMK, CIP, LVX	<i>aac(3')-II</i>	-
KP4	GEN	-	-
KP5	-	<i>aac(3')-II,</i>	-
KP6	-	<i>aac(3')-II, ant(3'')-I</i>	-
KP7	-	<i>aac(3')-II</i>	-
KP8	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-

KP9	GEN, AMK	<i>aac(3')-II, ant(3'')-I, aac(6')-Ib-cr</i>	-
KP10	GEN	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP11	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP12	-	<i>aac(3')-II,</i>	-
KP13	GEN, AMK, CIP, LVX	<i>aac(3')-II, aac(6')-Ib-cr</i>	+
KP14	-	<i>aac(3')-II,</i>	-
KP15	-	<i>aac(3')-II,</i>	-
KP16	GEN, AMK, CIP	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	+
KP17	GEN, AMK	<i>aac(3')-II, ant(3'')-I, aac(6')-Ib-cr</i>	-
KP18	-	<i>aac(3')-II,</i>	-
KP19	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP20	CIP, LVX	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP21	-	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP22	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP23	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP24	GEN, CIP	<i>aac(3')-II, ant(3'')-I</i>	-
KP25	-	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP26	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP27	GEN, AMK, CIP	<i>aac(3')-II, ant(3'')-I, aac(6')-Ib-cr</i>	+
KP28	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP29	-	<i>aac(3')-II, aac(6')-Ib-cr</i>	-
KP30	GEN, AMK, CIP, LVX	<i>aac(3')-II, aac(6')-Ib-cr</i>	+
KP31	GEN, AMK, CIP, LVX	<i>aac(3')-II, ant(3'')-I, aph(3')-VI</i>	-
KP32	GEN	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP33	CIP, LVX	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP34	-	<i>aac(3')-II, aph(3')-VI</i>	-
KP35	-	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP36	GEN, AMK	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP37	-	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP38	GEN, AMK, LVX	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	+
KP39	-	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP40	GEN, AMK	<i>aac(3')-II, , aac(6')-Ib-cr</i>	-
KP41	CIP	<i>aac(3')-II, ant(3'')-I, aph(3')-V, aac(6')-Ib-cr</i>	-
KP42	-	<i>aac(3')-II, , aac(6')-Ib-cr</i>	-
KP43	GEN, AMK	<i>aac(3')-II, , aac(6')-Ib-cr</i>	-
KP44	GEN, AMK	<i>aac(3')-II, , aac(6')-Ib-cr</i>	-
KP45	GEN, AMK, CIP	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	+
KP46	GEN, AMK, LVX	<i>aac(3')-II, , aac(6')-Ib-cr</i>	+
KP47	GEN, AMK, CIP, LVX	<i>aac(3')-II, ant(3'')-I, aph(3')-VI, aac(6')-Ib-cr</i>	+
KP48	GEN, AMK	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	-
KP49	GEN, AMK, CIP, LVX	<i>aac(6')-Ib-cr</i>	+
KP50	GEN, AMK, CIP, LVX	<i>aac(3')-II, aph(3')-VI, aac(6')-Ib-cr</i>	+

KP: *Klebsiella pneumoniae*, GEN: Gentamicin, AMK: Amikacin, CIP: Ciprofloxacin, LVX: Levofloxacin, aac: aminoglycoside acetyltransferase, ant: aminoglycoside nucleotidyltransferase, aph: aminoglycoside phosphotransferase, aac(6')-Ib-cr: aminoglycoside 6'-N-acetyltransferase-ciprofloxacin variant, (+): Positive result, (-): Negative result.

The results of DNA sequencing in the present study exhibited the presence of point mutations in the selected isolates (Figures-5,6, and 7). Some point mutations were observed to be of transition type, in which substitution occurs to pyrimidine with another pyrimidine or purine with another purine, whereas others were of transversion type, with replacement of pyrimidine with purine and vice versa [27]. Furthermore, the occurrence of frameshift mutation was observed in the DNA sequence of *ant(3'')-I* gene that led to the insertion of the triple CTT in the mutant sequence (Figure-7). The translation of protein revealed that some point mutations were silent, in which the amino acid sequences were not affected, but one point mutation was observed to be a missense mutation in which one amino acid is replaced with another, as illustrated in Table-4. Besides, the frameshift mutation involved the addition of glutamic acid to the amino acid sequence of *ant(3'')-I* gene. The missense and frameshift mutations may have contributed to the elevated MICs values of amikacin and gentamicin through their role in reducing the binding of the aminoglycoside molecule to the bacterial ribosome,

leading to subsequent failure of protein synthesis through mistranslation and proofreading rejection [4,6].

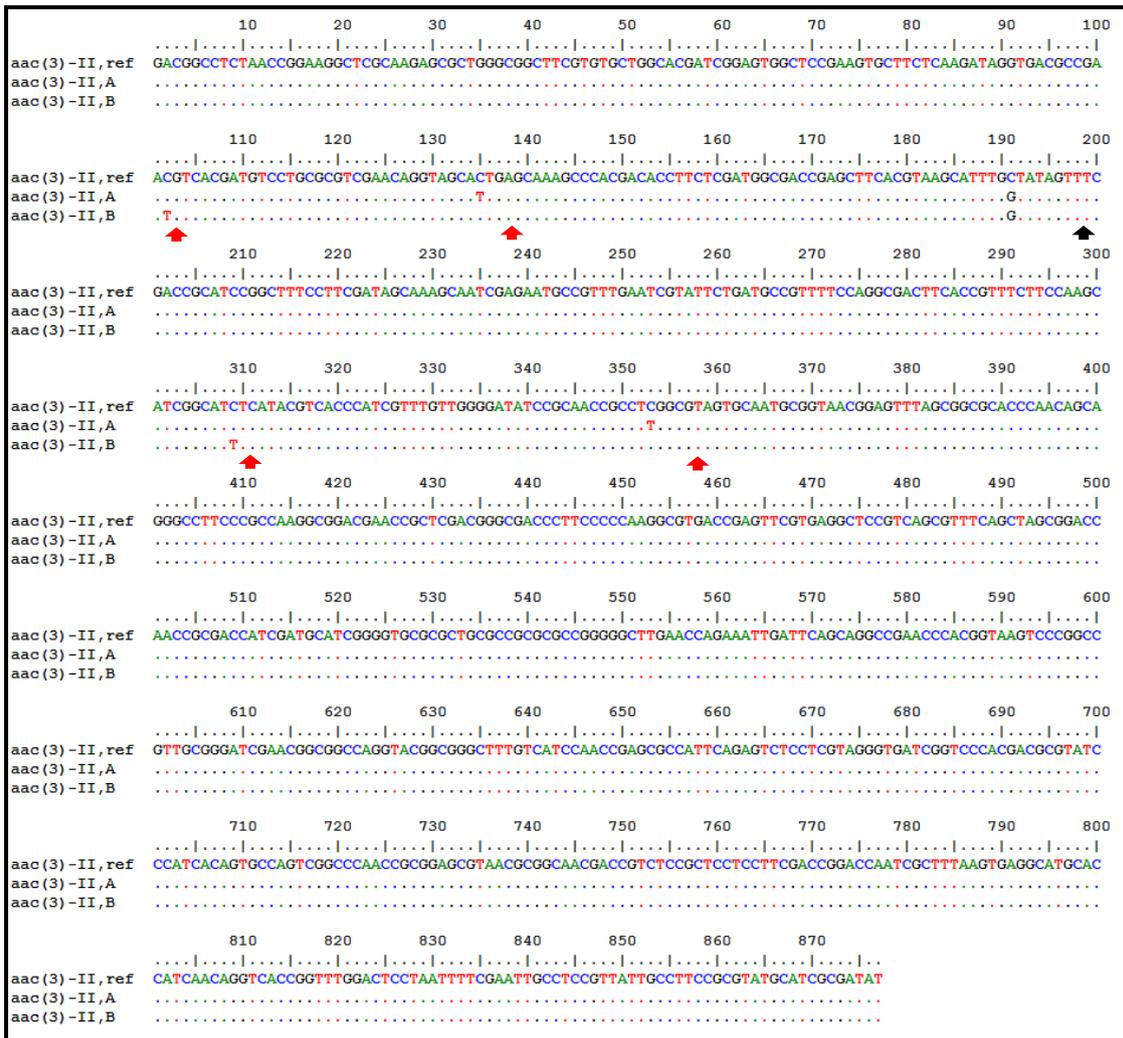


Figure 5-DNA Sequences alignment of the observed native strains with their corresponding reference sequences of the 877 bp amplicon of *aac(3')-II* gene (GenBank accession number CP025143.1). *aac(3')-II-A*: KP43, *aac(3')-II-B*: KP9.

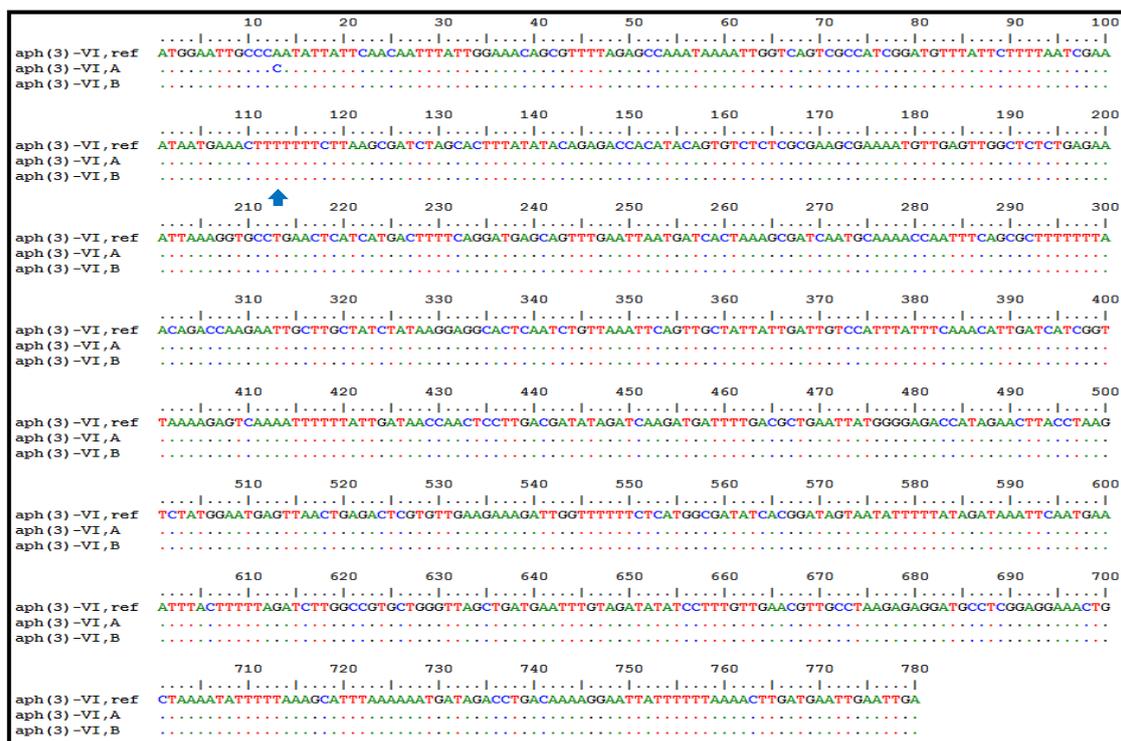


Figure 6-DNA Sequences alignment of the observed native strains with their corresponding reference sequences of the 780 bp amplicon of *aph(3')-VI* gene (GenBank accession number CP025517.1). *aph(3')-VI-A*: KP31, *aph(3')-VI-B*: KP45.

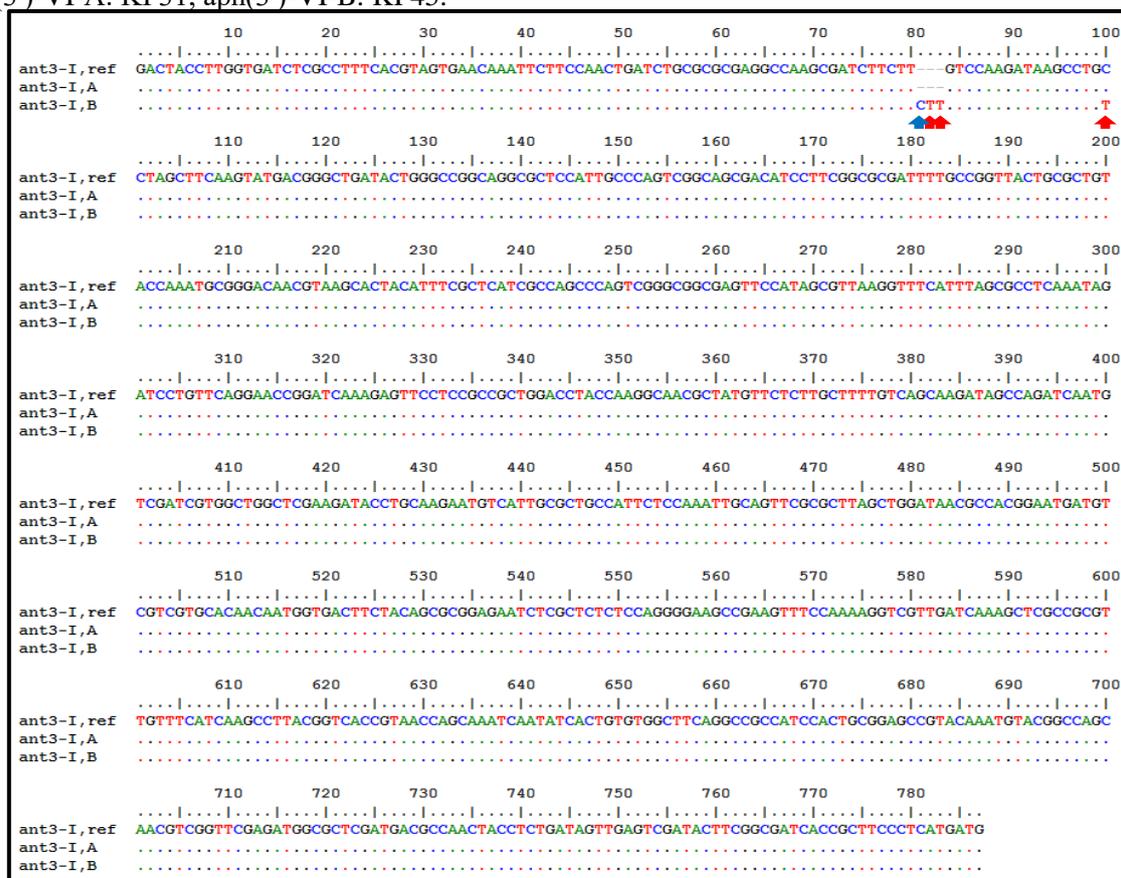


Figure 7-DNA Sequences alignment of the observed native strains with their corresponding reference sequences of the 787 bp amplicon of *ant(3'')-I* gene (GenBank accession number CP025517.1). *ant(3'')-I-A*: KP17, *ant(3'')-I-B*: KP27.

Table 4-The observed mutations in the DNA sequence of AMEs-encoding genes and amino acid substitutions of the selected *Klebsiella pneumoniae* isolates.

Isolate	Gene	DNA base substitution (Native>Allele)	Position in the reference DNA	Type of substitution	Type of point mutation	Amino acid substitution
KP9	<i>aac(3')-II</i>	C>T	141530	Transition	Silent	-
KP43	<i>aac(3')-II</i>	C>T	141563	Transition	Silent	-
KP43, KP9	<i>aac(3')-II</i>	C>G	141619	Transversion	Silent	-
KP9	<i>aac(3')-II</i>	C>T	141737	Transition	Silent	-
KP43	<i>aac(3')-II</i>	C>T	141781	Transition	Silent	-
KP31	<i>aph(3')-VI</i>	A>C	23353	Transversion	Missense	N>H
KP27	<i>ant(3'')-I</i>	CTT	9094-9095	Insertion	Frameshift	E
KP27	<i>ant(3'')-I</i>	C>T	9111	Transition	Silent	-

KP: *Klebsiella pneumoniae*, *aac*: aminoglycoside acetyltransferase, *aph*: aminoglycoside phosphotransferase, *ant*: aminoglycoside nucleotidyltransferase, C: Cytosine, T: Thymine, G: Guanine, A: Adenine, E: Glutamic acid, N: Asparagine, H: Histidine.

Conclusions

Overall, the present study concluded that AACs were the most prevalent AMEs among the investigated *K. pneumoniae* isolates, followed by APHs and ANTs. The increasing spread of AMEs-encoding genes reduced the susceptibility for amikacin and gentamicin, making the control on MDR *K. pneumoniae* inevitably very difficult. The co-resistance pattern for aminoglycosides and fluoroquinolones was found at low prevalence among *K. pneumoniae* isolates, but it was highly associated with the presence of the *aac(6')-Ib-cr* gene. Since *K. pneumoniae* serves as a worldwide source for the dissemination of AMEs-encoding genes and PMQR, even to other genera of non-Enterobacteriaceae, further surveys should be accomplished in this field to control the development of antibiotic resistance as much as possible, given the limited options of antimicrobial therapy.

Acknowledgment

We thank the administration of Medical City in Baghdad for their assistance over the period of samples collection. We also acknowledge ASCo learning center for supporting the molecular aspect of this study.

References

- Brenner, D.J., Krieg, N.R., and Staley, J.T. **2005**. Bergey's Manual of Systematic Bacteriology. Volum Two. Part B. 2nd ed. USA. Springer.
- Martin, R.M. and Bachman, M.A. **2018**. Colonization, Infection, and the Accessory Genome of *Klebsiella pneumoniae*. *Frontiers in Cellular and Infection Microbiology*, **8** (4): doi:10.3389/fcimb.2018.00004.
- Navon-Venezia, S., Kondratyeva, K., and Carattoli, A. **2017**. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiology Reviews*, **41** (3):252-75.
- Serio, A.W., Magalhaes, M.L., Blanchard, J.S., and Connolly, L.E. **2017**. Aminoglycosides: Mechanisms of Action and Resistance. In: Douglas L. Mayers, Jack D. Sobel, Marc Ouellette, Keith S. Kaye, and Dror Marchaim (eds), Antimicrobial Drug Resistance, Mechanisms of Drug resistance. Volum 1. 2nd ed. Switzerland: Springer, 213-29.
- Jacoby, G.A. **2017**. Plasmid-Mediated Quinolone Resistance. In: Douglas L. Mayers, Jack D. Sobel, Marc Ouellette, Keith S. Kaye, and Dror Marchaim (eds), Antimicrobial Drug Resistance, Mechanisms of Drug resistance. Volum 1. 2nd ed. Switzerland: Springer, 265-8.

6. Krause, K.M., Serio, A.W., Kane, T.R. and Connolly, L.E. **2016**. Aminoglycosides: An Overview. *Cold Spring Harbor Perspectives in Medicine*, **6** (6): pii: a027029. doi: 10.1101/cshperspect.a027029.
7. Raheison, S., Jove, T., Gaschet, M., Pinault, E., Tabesse, A., and Torres, C. **2017**. Expression of the *aac(6′)-Ib-cr* Gene in Class 1 Integrons. *Antimicrobial Agents and Chemotherapy*, **61** (5): e02704-16.
8. Tille, P.M. **2017**. Bailey and Scott’s Diagnostic Microbiology. 14th ed. China. Elsevier, Inc.
9. Gundersen Health System. **2018**. Vitek 2 Compact-Identification and Susceptibility Testing. Standard Operating Procedure. USA.
10. CLSI. **2017**. Performance Standard for Antimicrobial Susceptibility Testing. 27th ed. CSLI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute.
11. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. **2003**. Current Protocols in Molecular Biology. USA. Wiley and Sons, Inc.
12. Hu, X., Xu, B., Yang, Y., Liu, D., Yang, M., and Wang, J. **2013**. A high throughput multiplex PCR assay for simultaneous detection of seven aminoglycoside-resistance genes in Enterobacteriaceae. *BMC Microbiology*, **13** (58): doi: 10.1186/1471-2180-13-58.
13. Magill, S. S., Edwards, J. R., Bamberg, W., Beldavs, Z. G., Dumyati, G., Kainer, M. A., Lynfield, R., Maloney, M., McAllister-Hollod, L., Nadle, J., Ray, S. M., Thompson, D. L., Wilson, L. E., and Fridkin, S. K. **2014**. Emerging Infections Program Healthcare-Associated Infections and Antimicrobial Use Prevalence Survey Team (2014). Multistate point-prevalence survey of health care-associated infections. *The New England journal of medicine*, **370** (13): 1198–208.
14. Abd Al-Rahman, R.M. and Al-Aubydi, M.A. **2015**. Determination of the Relationship between Some Genetic Aspects with the Capsule Formation for Pathogenic *Klebsiella pneumoniae* Serotypes K1 and K2. *Iraqi Journal of Science*, **56** (2B).1385-93.
15. Adeosun, I.J., Oladipo, E.K., Ajibade, O.A., Olotu, T.M., Oladipo, A.A., Awoyelu, E.H., Alli, O.A.T., and Oyawoye, O.M. **2019**. Antibiotic Susceptibility of *Klebsiella pneumoniae* isolated from Selected Tertiary Hospitals in Osun State, Nigeria. *Iraqi Journal of Science*, **60** (7).1423-29.
16. Zafer, M.M., El-Mahallawy, H.A., and Abdulhak, A. **2019**. Emergence of colistin resistance in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from cancer patients. *Annals of Clinical Microbiology and Antimicrobials*, **18** (40): <https://doi.org/10.1186/s12941-019-0339-4>
17. Mokhtari, H., Eslami, G., Zandi, H., Dehghan-Banadkouki, A., and Vakili, M. **2018**. Evaluating the Frequency of *aac(6′)-IIa*, *ant(2′′)-I*, *intI1*, and *intI2* Genes in Aminoglycosides Resistant *Klebsiella pneumoniae* Isolates Obtained from Hospitalized Patients in Yazd, Iran. *Avicenna Journal of Medical Biotechnology*, **10** (2):115–9.
18. Liu, Y., Du, F., and Xiang, T. **2019**. High Prevalence of Plasmid-Mediated Quinolone Resistance Determinants Among Serotype K1 Hypervirulent *Klebsiella pneumoniae* Isolates in China. *Microbial Drug Resistance*, **25** (5): 681–9.
19. Albaayit, S.F.A., Al-Khafaji, A.S.K., and Radif H.M. **2018**. Investigation of plasmid-associated fluoroquinolone resistance in nosocomial *Pseudomonas aeruginosa* isolated from infected burn wounds. *Journal of Biological Sciences*, **18** (8): 514-9.
20. El-Badawy, M.F., Tawakol, W.M., El-Far, S.W., Maghrabi, I.A., Al-Ghamdi, S.A., Mansy, M.S., Ashour, M.S., and Shohayeb, M.M. **2017**. Molecular Identification of Aminoglycosides-Modifying Enzymes and Plasmid-Mediated Quinolone Resistance Gene among *Klebsiella pneumoniae* clinical isolates recovered from Egyptian patients. *International Journal of Microbiology*, 2017: 8050432. doi: 10.1155/2017/8050432.
21. Lotfollahi, L., Samadi, N., Hosainzadegan, H., and Qomi, M.A. **2015**. Prevalence of *acc(3′)-IIa* and *acc(6′)-Ib* genes incidence involved in aminoglycosides resistance in *Klebsiella pneumoniae*

- isolated from clinical samples in urmia hosopital, Iran. *American Journal of Pharmtech Reaserch*, **5** (5): 326-34.
22. Almaghrabi, R., Clancy, C.J., Doi, Y., Hao, B., Chen, L., and Shields, R.K. **2014**. Carbapeneme-Resistant *Klebsiella pneumoniae* Strains Exhibited Diversity in Aminoglycosides-Modifying Enzymes, Which Exert Different Effects on Plazomicin and other agents. *Antimicrobial Agents and Chemotherapy*, **58** (8): 4443-51.
 23. Landman, D., Babu, E., Shah, N., Kelly, P., Backer, M., and Bratu, S. **2010**. Activity of a novel aminoglycosides, ACHN-490, against clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from New York City. *The Journal of Antimicrobial Chemotherapy*, **65** (10): 2123-7.
 24. Hammad, H.A., Hadiya, S., EL-Feky, M.A., and Aly, S.A. **2017**. Co-occurrence of Plasmid-Mediated Quinolone Resistance and Carbapenemases in *Klebsiella pneumoniae* isolated in Assiut, Egypt. *Egyptian Journal of Medical Micrbiology*, **26** (4): (Abst.).
 25. Yan, J., Pu, S., Jia, X., Xu, X., Yang, S., and Shi, J. **2017**. Multidrug Resistance Mechanisms of Carbapenem Resistant *Klebsiella pneumoniae* strains isolated in Chongqing, China. *Annals of Laboratory Medicine*, **37** (5): 398-407.
 26. Schechner V., Temkin E., Harbarth S., Carmeli Y., and Schwaber M.J. **2013**. Epidemiological interpretation of studies examining the effect of antibiotic usage on resistance. *Clinical Microbiology Reviews*, **26** (2): 289–307.
 27. Snyder, L., Peters, J.E., Henkin, T.M., and Champness, W. **2013**. Molecular Genetics of Bacteria. 4th ed. Washington. ASM press.