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Role of *oqxA* and *oqxB* Genes in the Development of Multidrug Resistant Phenotype among Clinical *Klebsiella pneumoniae* Isolates from Various Cases

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Abstract

This study investigated the prevalence of *oqxA* and *oqxB* genes and their effective roles in the development of multidrug resistant (MDR) phenotype among clinical isolates of *Klebsiella pneumoniae*. Out of 150 clinical samples, 50 (33%) isolates were recognized as *K. pneumoniae* according to the morphological and biochemical properties. The minimum inhibitory concentrations (MICs) assay revealed that the resistance values of the isolates were 43 (86%) against ceftriaxone (4- ≥64 µg/ml), 42 (84%) against ceftazidime (16- ≥64 µg/ml), 41 (82%) against cefepime (≥16 µg/ml), 21 (42%) against ertapenem (≥8 µg/ml), 18 (36%) against imipenem (4- ≥16 µg/ml), 15 (30%) against ciprofloxacin (≥4 µg/ml), 11 (22%) against levofloxacin (≥8 µg/ml), 45 (90%) against nitrofurantoin (128- ≥512 µg/ml), 36 (72%) against trimethoprime-sulfamethoxazole (≥320 µg/ml), and 4 (8%) against tigecycline (≥8 µg/ml). Genotype detection revealed that *oqxA* was found in 48 (96%) of *K. pneumoniae* isolates, whereas *oqxB* was found in 6 (12%) isolates. The MDR phenotype was observed in 40 (80%) isolates, of which 38 (95%) were harbored *oqxA* and/or *oqxB* genes. DNA sequencing of *oqxA* revealed the presence of three silent mutations. The phylogenetic tree of *oqxA* variants showed a significant deviation of these variants from *K. pneumoniae* species. The high prevalence of *oqxA* among *K. pneumoniae* isolates may contribute to the reduction of their susceptibility to multiple antimicrobial agents.

Keywords: *Klebsiella pneumoniae*, MDR, *oqxA*, *oqxB*, Phylogenetic tree.

دور جينات *oqxA* و *oqxB* في تطور نمط المقاومة المتعددة في عزلات سريرية لبكتيريا *Klebsiella pneumoniae* من حالات مختلفة

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الخلاصة

تحرت هذه الدراسة عن تواجد الجينين *oqxA* و *oqxB* و تأثيرهما الفعال في تطور نمط المقاومة المتعددة MDR في عزلات سريرية لبكتيريا *Klebsiella pneumoniae*. تم الحصول على 50 (33%) عزلة من بكتريا *K. pneumoniae* من 150 عينة سريرية ، اذ شخصت حسب الصفات المظهرية و

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الكيموحيوية. اظهرت نتائج التراكيز المثبطة الدنيا MICs ان قيم المقاومة للعزلات كانت 43 (86%) لمضاد ceftriaxone (4-≥64 مايكروغرام\مل) و 42 (84%) لمضاد ceftazidime (16-≥64 مايكروغرام\مل) و 41 (82%) لمضاد cefepime (≥16 مايكروغرام\مل) و 21 (42%) لمضاد ertapenem (≥8 مايكروغرام\مل) و 18 (36%) لمضاد imipenem (4 -≥16 مايكروغرام\مل) و 15 (30%) لمضاد ciprofloxacin (≥4 مايكروغرام\مل) و 11 (22%) لمضاد levofloxacin (≥8 مايكروغرام\مل) و 45 (90%) لمضاد nitrofurantoin (128- ≥512 مايكروغرام\مل) و 36 (72%) لمضاد trimethoprime-sulfamethoxazole (≥320 مايكروغرام\مل) و 4 (8%) لمضاد tigecycline (≥8 مايكروغرام\مل). اظهرت نتائج التشخيص الجيني ان الجين *oqxA* كان موجودا ضمن 48 (96%) من عزلات بكتريا *K. pneumoniae* ، في حين ان الجين *oqxB* كان موجودا في 6 (12%) من العزلات. ان نمط المقاومة المتعددة MDR تم تشخيصه ضمن 40 (80%) من العزلات، من ضمنها 38 (95%) كانت تمتلك الجين *oqxA* او *oqxB* او كلاهما. اظهر تحليل تتابع الحامض النووي DNA للجين *oqxA* وجود ثلاث طفرات صامتة. ان الشجرة التطورية لتغايرات الجين *oqxA* اظهرت ان هناك انحراف ملحوظ لهذه التغايرات عن النوع *K. pneumoniae* . ان تواجد الجين *oqxA* في عزلات بكتريا *K. pneumoniae* بنسبة عالية قد ساهم في اختزال حساسيتها لعدة مضادات حيوية.

Introduction

Klebsiella pneumoniae is a Gram negative, non-motile, lactose fermenter, facultative anaerobic bacillus with a prominent polysaccharide capsule and belongs to the family Enterobacteriaceae [1]. It is an opportunistic pathogen that can colonize the mucosal surfaces of humans and cause serious nosocomial and community acquired infections, including urinary tract infections (UTIs), pneumonia, bacteremia, wound and burn infections, liver abscess, meningitis, cellulitis, and myositis [2].

Antimicrobial resistance becomes a worldwide problem in modern medicine that threatens public health. Since *K. pneumoniae* is a hospital-associated pathogen that is continuously treated with multiple antibiotics, it developed resistance abilities through multiple mechanisms against most common antibiotics in clinical usage [3]. This led to the emergence of multidrug resistant (MDR) *K. pneumoniae* that is responsible for high rates of morbidity and mortality due to the limited options of clinical treatment [3].

Efflux pumps are one of the resistance mechanisms employed by *K. pneumoniae* to be involved in both intrinsic and acquired resistance to antibiotics by decreasing intracellular concentrations of antibiotics and promoting accumulation of mutations [4]. The OqxAB is the predominant efflux pump found in *K. pneumoniae* that confers resistance against multiple antibiotics, including quinoxalines, quinolones, fluoroquinolones, chloramphenicol, trimethoprime, nitrofurantoin, and tigecycline [5].

The OqxAB efflux pump belongs to the resistance nodulation division (RND) family and consists of OqxA as a periplasmic part and OqxB as a transmembrane protein [4]. The *oqxAB* gene was first identified in 2003 on the pOLA52 plasmid in *Escherichia coli* from swine manure in Denmark [6]. Since then, *oqxAB* has been increasingly detected among *K. pneumoniae* as one of the plasmid mediated quinolone resistance (PMQR) mechanisms over the past decades [4,5].

The expression of the OqxAB efflux pump is regulated by RarA (regular of antibiotic resistance A) as an activator and OqxR (GntR-type transcriptional repressor) as a repressor [7]. Transposition of *oqxAB* gene from chromosome to plasmid has the ability to increase the expression level of OqxAB efflux pump in more than 80-fold, leading to the expansion of the MDR phenotypes [5,7].

This study aimed to investigate the prevalence of *oqxA* and *oqxB* genes and their role in the development of the MDR phenotypes in clinical isolates of *K. pneumoniae*.

Materials and Methods

Collection of Samples

A total of 150 clinical samples was recovered from patients who suffered from UTIs, bacteremia, pneumonia, burns, and wounds infections from different hospitals in Baghdad. The collection of samples was performed during the period from July to October in 2018, following the instructions of the ethics committee of the Ministry of Health in Baghdad according to the official approval numbered 31864.

Bacterial Isolation and Identification

The clinical samples were cultured 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 [8]. Then, the pure colonies were selected and identified by several biochemical assays, including oxidase, catalase, and IMVIC tests [8]. The Vitek 2 Compact System (BioMerieux, France) was used to confirm the classic identification of bacteria [9].

Antimicrobial Susceptibility Test

The antimicrobial susceptibility was tested by determining the minimum inhibitory concentrations (MICs) using Vitek 2 AST-GN cards (BioMerieux, France) by Vitek 2 Compact System [9]. All *K. pneumoniae* isolates were tested for susceptibility to Ceftazidime (1, 2, 8, 32 µg/ml), Ceftriaxone (1, 2, 8, 32 µg/ml), Cefepime (2, 8, 16, 32 µg/ml), Ertapenem (0.5, 1, 6 µg/ml), Imipenem (1, 2, 6, 12 µg/ml), Ciprofloxacin (0.5, 2, 4 µg/ml), Levofloxacin (0.25, 0.5, 2, 8 µg/ml), Nitrofurantoin (16, 32, 64 µg/ml), Trimethoprime-Sulfamethoxazole (1/19, 4/76, 16/304 µg/ml), and Tigecycline (0.75, 2, 4 µg/ml). Several pure colonies from overnight *K. pneumoniae* isolates on MacConkey agar 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 for AST-GN cards [9]. The results were interpreted following CLSI [10], except for tigecycline which was approved according to FDA because no CLSI breakpoints are available for this drug [11].

Molecular Characterization of *oqxA* and *oqxB* Genes

DNA was extracted from overnight *K. pneumoniae* colonies that grew on MacConkey agar using Wizard Genomic DNA Purification Kit (Promega, USA) according to the information from the manufacturing company. The concentrations and purity of DNA were measured by the Nanodrop (BioDrop, UK) [12]. The extracted DNA was screened for *oqxA* and *oqxB* genes using the primers (Alpha DNA, USA) that are reported in Table-1. The lyophilized product of these primers was dissolved in sterilized deionized distilled water (ddDW) (Promega, USA) to obtain 100 pmol/µl and then diluted to 10 pmol/µl according to the information from the manufacturing company. The polymerase chain reaction (PCR) was performed using Thermal Cycler (BioRad, USA). The PCR reaction mixture (20 µl) consisted of 10 µl Go Taq Green Master Mix (Promega, USA), 2 µl template DNA (84.95 ng/µl), 1 µl of both forward and reverse primers, and 6 µl ddDW [12]. The PCR thermal cycler was programmed for *oqxA* and *oqxB* genes under the optimal conditions [13] that are reported in Tables-(2 and 3), respectively. The PCR amplification was verified by electrophoresis at 100 V for 80 min on 1% agarose gel stained with 0.5 µg/ml ethidium bromide in 1X TAE buffer (Promega, USA) using a DNA ladder (100-1500 bp), supplied by Promega (USA), as a molecular weight marker. The UV- Transilluminator (Major Science, Taiwan) was used for the observation of PCR products under 320nm UV light [12].

Table 1-Sequences of primers used in this study

Genes	Primer Sequence (5´- 3´)	Size (bp)	Reference
oqxA	F: CTCGGCGCGATGATGCT R: CCACTCTTCACGGGAGACGA	392	[13]
oqxB	F: TTCTCCCCCGGCGGGAAGTAC R: CTCGGCCATTTTGGCGCGTA	512	[13]

Oqx: Olaquindox, F: forward, R: reverse, bp: base pair.

Table 2-The optimal conditions for amplifying *oqxA* by PCR

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

Table 3-The optimal conditions for amplifying *oqxB* by PCR

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

DNA Sequencing

The sequencing of two PCR products that referred to the *oqxA* from both ends was done by Genetic Analyzer (Macrogen Inc., South Korea) according to the instructions of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. The result was compared with the reference database available at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). The sequencing results of the PCR products of the sequenced isolates were edited, aligned, and analyzed along with their respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA) (http://bioedit.software.informer.com/7.1/) [14]. Each observed variation was translated into amino acids in a reading frame corresponds to the reference amino acid sequences using the Expasy online program (http://web.expasy.org/translate/). The referring sequences were manually aligned side by side with their mutant counterparts [15].

Phylogenetic Tree Construction

The observed PCR amplicon variants of *oqxA* genetic loci were compared with the neighbor homologous sequences using NCBI-BLASTn suite (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/). The 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 (33%) were identified as *K. pneumoniae*. The bacterial isolates were found at a high prevalence in urine 15 (30%) followed by blood 12 (24%), whereas lower prevalence appeared in sputum and wounds 9 (18%), and finally burns 5 (10%). Several studies revealed that the most common site of *K. pneumoniae* infections is UTIs followed by bloodstream infections, pneumonia, and burns and wounds infections [2,16,17]. Beside that, other studies collected *K. pneumoniae* from other cases, including pus, stool, cerebrospinal fluid, catheters, and eye and ear swabs [18,19,20].

The MICs listed in Table-4 exhibited that imipenem and ertapenem were more effective against *K. pneumoniae* isolates than other β-lactams, in which 18 (36%) (MIC 4- ≥16 µg/ml) and 21 (42%) (MIC ≥8 µg/ml) of the isolates were resistant to these drugs, respectively. A previous study [21] demonstrated that all *K. pneumoniae* isolates were resistant for both agents. *K. pneumoniae* isolates showed high resistance values for other β-lactams that included ceftriaxone, ceftazidime, and cefepime, reaching 43 (86%) (MIC 4-≥64 µg/ml), 42 (84%) (MIC 16- ≥64 µg/ml), and 41 (82%) (MIC ≥16 µg/ml), respectively. The resistance rates gained by another study [19] against these three agents were relatively in line with those of this study. The production of extended spectrum beta-lactamases (ESBLs), AmpC- β-lactamases, and carbapenemases is the main reason for the high level of resistance against cephalosporins and carbapenems [22]. The increasing use of carbapenems, as one of the last resorts in the clinical treatments worldwide resulted in the dissemination of *K. pneumoniae* carbapenemases (KPC) in the health care settings, causing a potential threat to the public health [23]. The challenge was more aggravated when carbapenemases were found to confer resistance not only to carbapenems but also to non β-lactams, particularly aminoglycosides and fluoroquinolones [22,23].

Table 4-MICs Values of *Klebsiella pneumoniae* isolates

Antibiotics	Resistant Values of MICs (µg/ml)	Resistant Isolates No. (%)
Ertapenem	≥8	21 (42)
Imipenem	4- ≥16	18 (36)
Ceftriaxone	4- ≥64	43 (86)
Ceftazidime	16- ≥64	42 (84)
Cefepime	≥16	41 (82)
Ciprofloxacin	≥4	15 (30)
Levofloxacin	≥8	11 (22)
Nitrofurantoin	128- ≥512	45 (90)
Trimethoprime-Sulfamethoxazole	≥320	36 (72)
Tigecycline	≥8	4 (8)

Regarding fluoroquinolones, 15 (30%) of *K. pneumoniae* isolates were resistant to ciprofloxacin (MIC ≥4 µg/ml), while 11 (22%) were resistant to levofloxacin (MIC ≥8 µg/ml). Higher resistance rates for both agents were obtained by an earlier investigation [16]. The resistance for these antibiotics is mediated by the mutations in the topoisomerases encoding genes (*gyrA* and *parC*), acquisition of PMQR, and alternations of cell permeability through the loss of porins and the overexpression of the MDR efflux pumps OqxAB [4,5]. The OqxAB efflux pumps mediate low level fluoroquinolones resistance, enabling the bacteria to survive in the face of these drugs as long as possible and enhancing the chromosomal mutations in the *gyrA* and *parC* that are associated with high level fluoroquinolones resistance [5,7].

Nitrofurantoin was the least effective antibiotic against *K. pneumoniae* isolates, whereas tigecycline was more effective with values of 45 (90%) (MIC 128-≥512 µg/ml) and 4 (8%) (MIC ≥8 µg/ml), respectively. Moreover, 36 (72%) (MIC ≥320 µg/ml) of the isolates were resistant for Trimethoprime-Sulfamethoxazole. The resistant rates of this study were higher than those reported by a previous study [24] for both Nitrofurantoin and Trimethoprime-Sulfamethoxazole and by another study [19] for tigecycline. In addition to fluoroquinolones, the MDR efflux pump OqxAB has been linked with the cross-resistance phenotype, in which this pump contributes to the reduction of susceptibility to multiple agents, including nitrofurantoin, trimethoprime-sulfamethoxazole, chloramphenicol, and tigecycline [5,7].

The MICs indicated that 40 (80%) of *K. pneumoniae* isolates were MDR. The bacterial isolates were considered MDR when they exhibited resistance to at least three or more unrelated agents [25]. Currently, MDR *K. pneumoniae* has become an escalating global threat in preventive medicine with the limited medical treatments and the difficulty of providing alternative findings [3]. The widely misused of the antibiotic therapy and horizontal transfer of the resistance genes participate in the emergence and development of MDR *K. pneumoniae* [3].

Genotypic screening revealed that *oqxA* was detected in 48 (96%) of *K. pneumoniae* isolates with a PCR product of 392 bp, whereas *oqxB* was found only in 6 (12%) of isolates with a PCR product of 512 bp, as shown in Figures-(1 and 2), respectively. The studies conducted by several researchers [19,26,27] showed similar high incidences of *oqxA* among the used *K. pneumoniae* isolates. In addition, a number of studies [19,28,29] exhibited that *oqxB* was more prevalent among the isolates than that in this study. The high prevalence of *oqxA* in *K. pneumoniae* isolates represents a potential reservoir for the spread of this gene [5]. The horizontal transfer and transposition of these genes from chromosome to plasmid enhance the ability to increase the level of MDR phenotypes [3].

As previously mentioned, MDR phenotype was observed among 40 (80%) of *K. pneumoniae* isolates, of these 38 (95%) harbored *oqxA* and/or *oqxB* (Table-5) that play an influential role in broaden bacterial resistance for various classes of antimicrobial drugs [4]. The correlation between phenotype (MICs) and genotype (PCR) of fluoroquinolones resistance revealed that 17 (34%) of *K. pneumoniae* isolates showed resistance against ciprofloxacin and/or levofloxacin, among which 16 (94%) possessed *oqxA* and/or *oqxB*, as illustrated in Table 5. Earlier studies [26,27,28,29] explained that the reduced susceptibility of the used *K. pneumoniae* isolates against multiple agents, particularly fluoroquinolones, may be due to the high prevalence of *oqxA* and/or *oqxB* among their isolates.

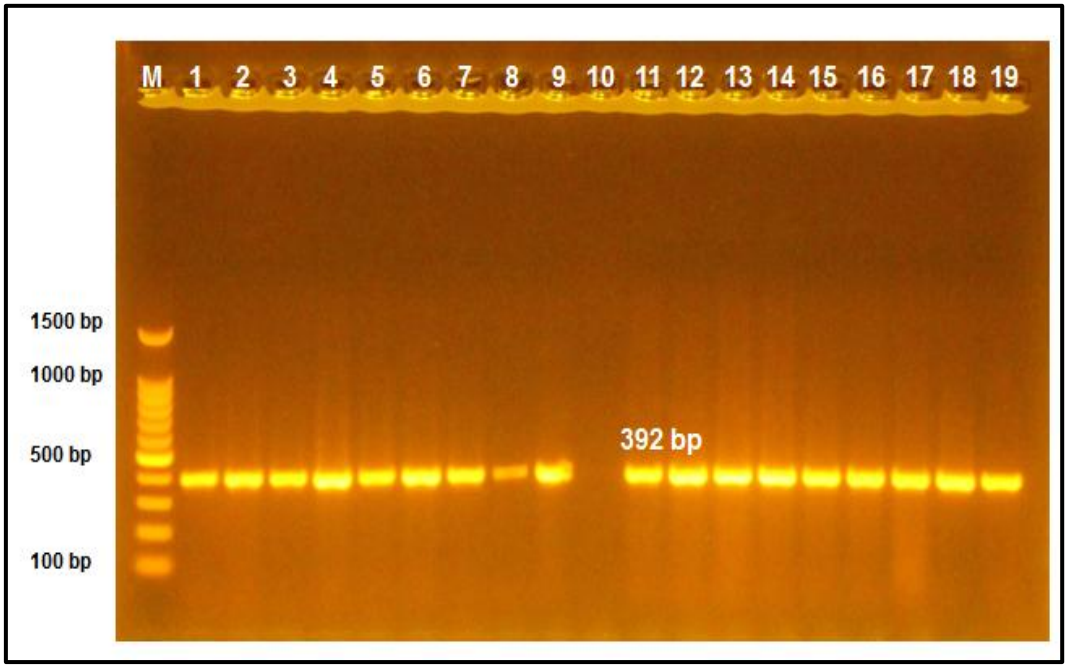

Figure 1- Gel electrophoresis of PCR products showing *oqxA* gene with 392 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 1-9 and 11-19 represent *Klebsiella pneumoniae* PCR positive isolates.

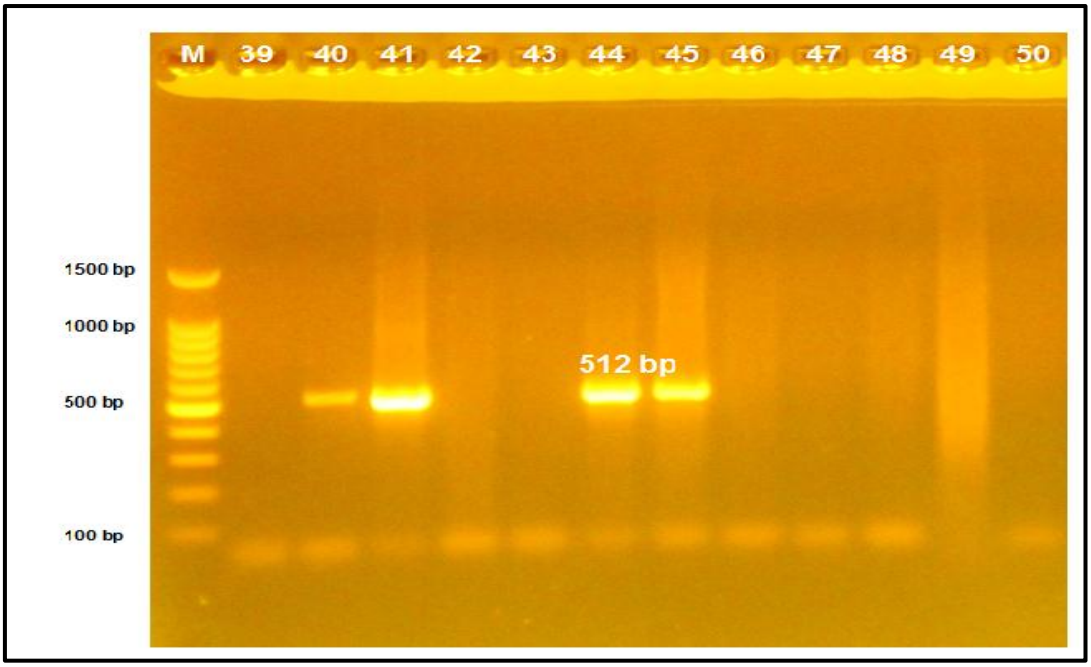

Figure 2- Gel electrophoresis of PCR products showing *oqxB* gene with 512 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 40,41,44 and 45 represent *Klebsiella pneumoniae* PCR positive isolates.

Table 5-Correlations of genotypic and resistant profiles in *Klebsiella pneumoniae* isolates

Isolate No.	Genotypic Profile	Resistant Profile	MDR Profile
KP1	*oqxA*	IMP, ETP, CAZ, CRO, FEP, NIT	+
KP2	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, NIT, SXT	+
KP3	*oqxA*	ETP, CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+

KP4	*oqxA*	ETP, CAZ, CRO, FEP, NIT, SXT	+
KP5	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP6	*oqxA*	NIT	-
KP7	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP8	*oqxA*	CAZ, CRO, FEP, NIT	-
KP9	*oqxA*	IMP, ETP, CAZ, CRO, FEP, NIT	+
KP10	-	ETP, CAZ, CRO, FEP, NIT, SXT	+
KP11	*oqxA*	NIT	-
KP12	*oqxA*	NIT	-
KP13	*oqxA* , *oqxB*	ETP, CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+
KP14	*oqxA*	NIT	-
KP15	*oqxA*	CAZ, CRO, NIT, SXT	+
KP16	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, NIT, SXT	+
KP17	*oqxA*	IMP, NIT	-
KP18	*oqxA* , *oqxB*	ETP, CAZ, CRO, FEP, NIT	+
KP19	*oqxA*	CAZ, CRO, FEP, NIT, TGC	+
KP20	*oqxA*	CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+
KP21	*oqxA*	NIT, SXT	-
KP22	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP23	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP24	*oqxA*	IMP, ETP, CAZ, CRO, FEP, SXT	+
KP25	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, NIT, SXT	+
KP26	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP27	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, NIT, SXT	+
KP28	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP29	*oqxA*	CAZ, CRO, FEP, SXT	-
KP30	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, LVX, NIT, TGC, SXT	+
KP31	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, LVX, SXT	+
KP32	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP33	*oqxA*	CAZ, CRO, FEP, CIP, LVX, NIT, TGC, SXT	+
KP34	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP35	*oqxA*	CRO, NIT	-
KP36	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP37	*oqxA*	-	-
KP38	*oqxA*	CAZ, CRO, FEP, LVX, NIT, TGC, SXT	+
KP39	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP40	*oqxA* , *oqxB*	IMP, ETP, CAZ, CRO, FEP, NIT, SXT	+
KP41	*oqxA* , *oqxB*	CAZ, CRO, FEP, CIP, NIT	+
KP42	*oqxA*	CAZ, CRO, FEP, NIT, SXT	+
KP43	*oqxA*	IMP, ETP, CAZ, CRO, FEP, SXT	+
KP44	*oqxA* , *oqxB*	IMP, ETP, CAZ, CRO, FEP, NIT	+
KP45	*oqxA* , *oqxB*	IMP, ETP, CAZ, CRO, FEP, CIP, NIT, SXT	+
KP46	*oqxA*	IMP, CAZ, CRO, FEP, LVX, NIT, SXT	+
KP47	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+
KP48	*oqxA*	IMP, ETP, CAZ, CRO, FEP, NIT, SXT	+
KP49	-	CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+
KP50	*oqxA*	IMP, ETP, CAZ, CRO, FEP, CIP, LVX, NIT, SXT	+

KP:*Klebsiella pneumoniae*, Oqx: Olaquindox, CIP: Ciprofloxacin, LVX: Levofloxacin, CAZ: Ceftazidime, CRO: Ceftriaxone, FEP: Cefepime, ETP: Ertapenem, IPM: Imipenem, TGC: Tigecycline, NIT: Nitrofurantoin, SXT: Trimethoprime-Sulfamethoxazole, MDR: Multidrug resistance, (+): Positive result, (-): Negative result.

DNA sequencing was applied on the oqxA-A (KP23) and oqxA-B (KP13) isolates in order to exhibit the MDR phenotype. The results presented in Figure-3 revealed that no mutation was observed in the DNA sequence of the isolate oqxA-A (KP23), although it showed resistance for more antibiotics that used in this study. This can be explained that isolate may be harbored other MDR encoded genes

that did not detect by this study. Otherwise, three point mutations were seen in the DNA sequence of the isolate oqxA-B (KP13). Two of these mutations were transition, in which led to the replacement of guanine with adenine (G>A) at the position 54 and cytosine with thymine (C>T) at the position 210. The third mutation was transversion that led to the replacement of guanine with cytosine (G>C) at the position 326. The translation of protein (Figure-4) revealed that these point mutations didn't cause any substitution in the amino acid sequences (silent mutations). The mutant DNA sequence of the isolate KP13 was registered in NCBI at the accession number LC381732.

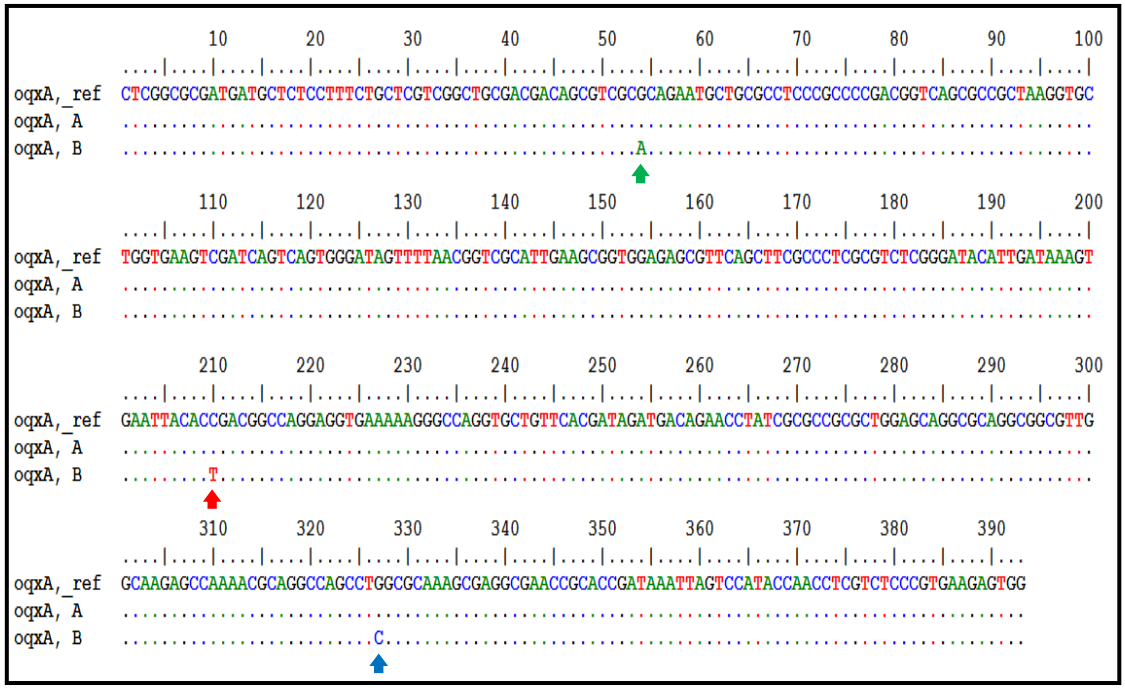

Figure 3-DNA Sequences Multiple Alignment of the oqxA-A (KP23) and oqxA-B (KP13) isolates with their corresponding reference sequences of the 392 bp amplicon of the *oqxA* gene (GenBank accession number CP027189.1). The observed polymorphisms were highlighted according to their position in the PCR products.

MSLQKTWGNIHLTALGAMMLSFLLVGCDDSVAQNAAPPAPTVSAAKVLVKSISQWDSFNGR
IEAVESVQLRPRVSGYIDKVNYTDGQEVKKGQVLFTIDDRTYRAALEQAQAALARAKTQASL
AQSEANRTDKLVHTNLVSREEWEQRRSAAVQAQADIRAAQAAVDAAQLNLDFTKVTAPIDG
RASRALITSGNLVTAGDTASVLTTLVSQKTVYVYFDVDESTYLHYQNLARRGQGASSDNQAL
PVEIGLVGEEGYPHQGKVDFLDNQLTPSTGTIRMRALLDNSQRLFTPGLFARVRLPGSAEFKA
TLIDDKAVLTDQDRKYVYIVDKDGKAQRRDITPGRLADGLRIVQKGLNPGDSVIVDGLQKVF
MPGMPVNAKTVAMTSSATLN

Figure 4-Amino Acids Sequence Analysis of the mutant oqxA-B (KP13) isolate. The shaded region indicated the coding portions within the entire primary amino acid sequences of the protein.

The phylogenetic tree (Figure-5) indicated the presence of seven species all over scanned of *oqxA* variants sequence-related species. The total number of the aligned DNA sequences in this tree was 100. In relation to both *oqxA* variants, the comprehensive involved organisms were included *K. pneumoniae*, *Enterobacter hormaechei*, *Escherichia coli*, *Escherichia albertii*, *Salmonella enterica*, and *Salmonella typhimurium*. This tree did not provide enough information about the real identity of the local isolates of *K. pneumoniae*. This fact is reflected from the position occupied by the non-mutant oqxA-A (KP23) variant, in which this variant was positioned very close to three species, namely *Salmonella enterica* (MF418180.1), *Escherichia coli* (KY075659.1), and *K. pneumoniae* (CP014647.1), respectively. Furthermore, the mutations observed in oqxA-B (KP13) variants (g.54G>A, g.210C>T, and g.326G>C) did not go further than these three species. This sort of positioning adds another layer of confirmation about the limited power of *oqxA* fragment based tree in

providing concrete information about the actual identity of such microbiological identified *K. pneumoniae* local isolates [5,6]. Nevertheless, *oqxA* gene is found to be commonly located on the chromosome of *K. pneumoniae* isolates [4,5]. So, *K. pneumoniae* may constitute a potential reservoir for the spread of *oqxA* gene, posing a threat to public health [5]. Moreover, the phylogenetic tree constructed by [7] which included various types of Enterobacteriaceae, suggested that *oqxAB* was originated from the chromosome of *K. pneumoniae*.

Conclusion

The high prevalence of *oqxA* among *K. pneumoniae* isolates may contribute to reduce the bacterial susceptibility to multiple antimicrobial agents, leading to increase the selection of MDR phenotype among the studied isolates. *K. pneumoniae* may represent a potential reservoir for the spread of this gene.

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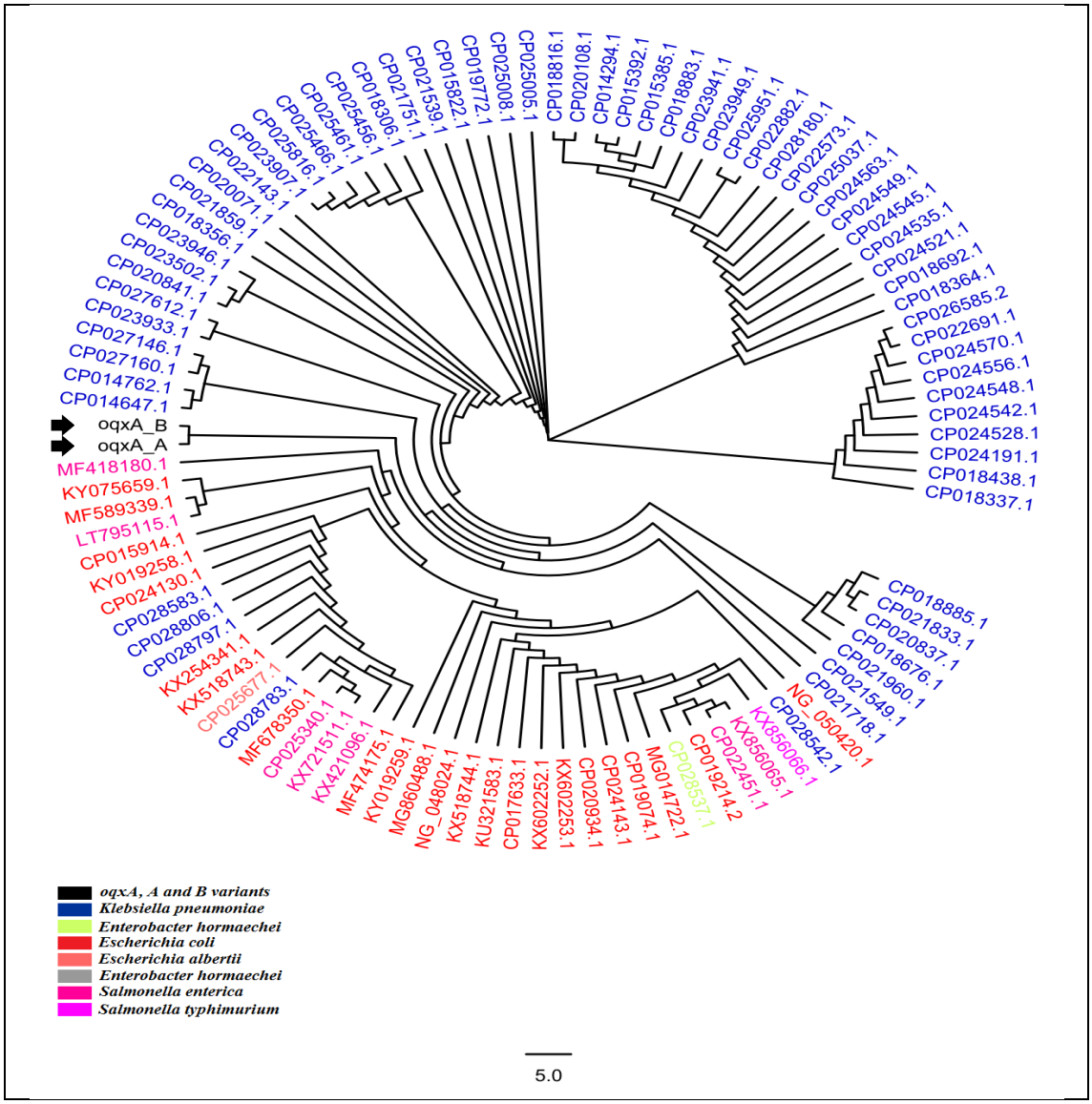

Figure 5-Phylogenetic tree of the 392 bp variants of *oqxA* genetic fragments for *K. pneumoniae* local isolates. The black color refers to the sequenced two variants (local isolates), while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to Genbank accession number of each referring species. The number "5.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

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