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Detection of *Ompk36* Mutations of *Klebsiella pneumoniae* and Determination their effect on Outer Membrane Permeability to Antibiotics

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Abstract*: OmpK36* is the major outer membrane porins of *Klebsiella pneumoniae*. This study was aimed to detection the some mutations in ompk36 and their effect in the outer membrane permeability to antibiotics and increase antibiotic susceptibility. This study was, performed on 250 clinical specimens collected from three major hospitals in wasit (Alkarama teaching hospital, Zahra teaching hospital and Al-Batool maternity hospital) between November 2021 to February 2022. The presence of antibiotic resistance was investigated by polymerase chain reaction (PCR) method. The purified PCR products of *ompk36* positive isolates were, sequenced to screen for mutations in *ompk36* gene. Among the types of mutations that were found in resistant isolates were the frameshift mutations, the mutations that occurred in the *ompk36* gene, they are as follows: i- substitution (197 N>S, 109 D >N, 195 A>G, and 197 N>D, 259 Silent mutation, 260 Y>N, 208 D>N, 293 A >G and 238 D>N)ii-Insertion(Ins 201 C, Ins 203 A, Ins 205 AC, and Ins 239 A) and deletion (Del 199 A, Del 101 A, Del 195 A, Del 196 C, Del 195 A).

Keywords: Ompk53, porin, *Klebsiella pneumoniae*

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Introduction

Klebsiella pneumoniae, a member of the family *Enterobacteriaceae*, is a part of the intestinal flora and it is isolated as the causative agent in harsh infections. It is an opportunistic microorganism, which shows a high tendency to acquire drug-resistant traits and can cause it a range of infections, including pneumonia, septicemia, meningitis, bacteremia, wound infections and purulent abscesses at different sites (1). *K. pneumoniae* is widely distributed in the respiratory, urinary, and gastrointestinal tracts of healthy people, most *K.pneumoniae* are hospital associated with a high fatality rate if incorrectly treated (2) Outer membrane porins are among the most recently discovered virulence factors. (3). Antibiotic influx across the outer membrane of *K. pneumoniae* is limited by chromosomal alteration of the main outer membrane porins, *OmpK35* and *OmpK36* (4). Outer membrane porins (OMPs) typically comprise trimers, which serve as water-filled protein channels for the transportation of hydrophilic substances through the external membrane such as β-lactams and fluoroquinolones. Porins are also a vector for phages and bacteriocins and play a major structural role in protecting

cell integrity with peptidoglycan and lipopolysaccharide (5). Therefore, the aim of this study was to identification of *K. pneumoniae* isolates from different clinical sources and determine the effect of mutations in antimicrobial resistance of common changes in major porins that reduce membrane permeability.

Materials and methods

Bacterial isolates

From November 2021 to February 2022, 250 samples were aseptically collected using sterile containers and transport swabs damped with normal saline from people of various source. All samples were Collected from Al Karama teaching hospital, Al Zahra teaching hospital and Al-Batool hospital for pediatrics and gynecology. *K. pneumoniae* isolates identified by standard biochemical tests, API 20E and confirmed by the VITEK-2 system and 16srRNA, where it was found that sixty-six isolates belong to *Klebsiella pneumoniae*.

Antibiotic susceptibility testing

The antibiotics susceptibility test was performed according to the Kirby-Bauer method and CLSI (2021) (6) criteria by using the disk-diffusion technique. The selected antibiotic discs were applied on the inoculated Mueller Hinton agar plate by sterile forceps and the plates were incubated at 37°C for 18-24 hours. Depending on CLSI (2021), the diameter of each inhibition zone (including the diameter of the disc) were measured with metric ruler, and recorded in mm. All *K.pneumoniae* isolates were tested against 15 antibiotics belonging to the 7 classes of antibiotics classified on their mechanism of action including : Penicillins , Penems , Quinolones , β-Lactams , Phenicols , Aminoglycosides and Nitrofurantoins .

Detection of *OMPK36* gene

OMPK36 gene was detected by a multiplex PCR technique. DNA was extracted using the boiling process described by Yamamoto *et al*. 1995 (7). Bacterial isolates were cultured on B.H.I agar, and incubated at 37oC for 24 h. Three loopful of old bacterial growth suspended in sterile 1X TE buffer (pH 8.0) or in 1ml sterile D.W. in Eppendorf tubes, mixed by vortex. The cell suspension was boiled in water bath at 95˚C for 10 minutes. A cell suspension was centrifuged for 5 min. at 10,000 rpm to separate the suspension. The supernatant that contain purified DNA was transferred to new Eppendorf tubes dispensed in 200 µl aliquots four repeated tubes and stored at -20˚C till use as DNA template.

Polymerase chain reaction

Primers were designed by Primer3Plus program software). Primers were supplied by to Macrogen Company (USA) as a lyophilized product. Lyophilized primer was dissolved in a DNase/RNase free water to give a final concentration of (100 pmol/µl) as stock solution. The primers were designed according to Primer3 web version 4.1.0 (online at website http://primer3.ut.ee) .The sequences of this primer were: Forward primer:5'ACGGCAACAAACTGGGA CTTC3';Reverse primer:5'-GACGGGTTTTTGTGGTCT G-3' and give the product size 208bp. PCR reactions were conducted under sterile conditions, using 25µl reaction mixture containing 12.5 µl of Go Taq® Green

Master Mix (Promega/USA) ,2µl of F-primer, 2µl R-primer,5µl DNA sample and 3.5 µl D.W. The standard cycle procedure was a 5- minute initial denaturation at 95°C for one cycle, then 35 cycles of 40sec of denaturation at 95 °C, 40sec of annealing 59 °C, 40sec extension at 72 °C and 5 minutes for final extension at 72°C.

Gel electrophoresis for PCR products

The PCR products and the DNA ladder were run by Electrophoresis system. 3µl of loading dye plus 9 µl of the ladder were mixed and loaded in the first well, also 9 µl of product were loaded in the next wells on 2% agarose gel (Promega/ USA). 1 gram of agarose were melted in 50 ml of 1X TBE buffer and run at 70 volt for 1hr.

PCR Products sequencing

The 25-µl PCR products of resist bacteria (58) samples of the analyzed *ompk36* gene and primers were, sent to Macrogen Company (USA) for sequencing the plus strand only. More information available on web site (http://www.macrogen.com). National Center for Biotechnology information (NCBI) / Basic local alignment search toll (blast) were used to detection the mutations in this gene (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM).

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Results discussion

Characteristics of clinical samples

K. pneumoniae 66 (26.4%) isolates were examined from 250 total clinical bacterial isolates at Al-Karama teaching hospital, Al-Zahra teaching hospital and Al-Batool hospital for pediatrics and gynecology, Wasit, Iraq. *K. pneumoniae* isolates were isolated from 125 Urine (50%), 70 Sputum (28%) and 55 Burn (22%) patients (Table 1).

NO.	Sample Source	No. of sample (%)
1	Urine	125
2	Sputum	70
3	Burn	55
Total		250

Table (1): Clinical samples source and numbers

Antibiotic susceptibility pattern

Sixty-six isolates of *K.pneumonia* from burn, sputum, and UTI patients were tested for antibiotic sensitivity by disk diffusion method based on measuring the diameter of the inhibition zone and comparing it with what was reported in the (CLSI, 2021). Results presented in Table (2) Show that *K. pneumoniae* isolates were resistant to

penicillins was very high at 100% of isolates being resistant to ampicillin this result was in agreement with Hostackai and Klokocnkovai (8), Because ampicillin is one of the most widely used antibiotics to treat urinary

tract infections (9). The widespread resistance in the Iraqi isolates constitutes a major challenge in the treatment of the disease. As regarding penicillins-β-lactamase inhibitors, amoxicillin-clavulanic acid (Augmentin) which is often used as an oral preparation, is clinically approved and is effective against penicillinase-producing bacteria. Several studies, Al-Obadi, 2014 (10), Al-Hasnawi, 2020 (11), demonstrated the competence of Augmentin against *K. pneumoniae* isolates with a resistance rate of (97.5%, 89.1%) respectively. While the current study showed less efficacy of this antibiotic in treating *K. pneumoniae* isolates with resistance rate (40.9%). This result is in agreement with the reported results by Balle'n, 2021(12) which were 40.54% and this may be due to the frequent use of this antibiotic to treat infections caused by Enterobacteriaceae. Most isolates exhibited resistance to extended-spectrum third generation cephalosporins, the rates resistance to cefotaxime, ceftazidime, ceftriaxone, and cefepime (4th-generation cephalosporin) were (46.9%) , (63.6%) , (37.8%), and (40.9%) , respectively. This finding is similar to that of Al-Timimi, 2021(13) that reported (42%) and (43%), of *K. pneumoniae* strains were resistant to Cefotaxime and Cefepime, respectively. In addition, the isolates showed a low rate of resistance to aminoglycosides including Tobramycin, amikacin and gentamicin were (66.6%), (1.5%) and (9.0%) respectively. In contrast, Carbapenems were still very active. Antibiotic susceptibility tests showed that Carbapenems (meropenem) were more effective than penicillins and cephalosporins as only (31.8%) of these isolates were resistant to meropenem. For Quinolones antibiotics, 19.6% and 22.7% of the isolates were resist to Nalidixic Acid and Ciprofloxacin, respectively. Similarly, Al-Obadi (2014) found that resistance to Ciprofloxacin was 20 %. While results for Ali *et al*., 2010 (14) was rather different from the data. They reported that about 72.22% of *Klebsiella* isolates were resistance to Ciprofloxacin.

Hashemi , 2014 (15) and Al-Mawasi (2018) demonstrated in their local study that the resistance rate of clinical *K. pneumoniae* isolates was 60.2% and 38.57%, respectively for Piperacillin isolates. As for the current study, it was found that the resistance rate is 50% the resistant rate of isolates to the remaining antibiotics was as follows: nitrofurantoin (6.0%) and chloramphenicol (4.5%) . which is considered the lowest resistance rate after amikacin. The relation between the Susceptibility to antibiotics of *K. pneumoniae* infection was found statistically highly significant ((P≤0.01).

Table (2): Antibiotic-susceptibility patterns of *K. pneumoniae* isolates (n = 66)

Class	Antibiotic	No. (%) of the isolates			P-value
		Resistance	Intermediate	Susceptible	
Penicillins	Ampicillin	66 (100%)	0 (0.0)	0(0.0)	0.0001 **
Penems	Meropenem	21 (31.8%)	1(1.5%)	44(66.6%)	0.0001 **
Quinolones	Nalidixic Acid	13(19.6%)	11(16.6%)	42(63.6%)	0.0001 **
	Ciprofloxacin	15(22.7%)	12 (18.1%)	39(59.0%)	0.0001 **
β-lactamase inhibitor combination	Amoxicillin-Clavulanic acid	27(40.9%)	2 (3.0%)	37(56.0%)	0.0001 **
β-lactams	Piperacillin	33(50%)	16(24.2%)	17(25.7%)	0.0093 **
Cephalosporin	Ceftazidime	42(63.6%)	1(1.5%)	23(34.8%)	0.0001 **
	Cefotaxime	31(46.9%)	1(1.5%)	34(51.5%)	0.0001 **
	Ceftriaxone	25(37.8%)	2(3.0%)	39(59.0%)	0.0001 **
	Cefepime	27(40.9%)	1(1.5%)	38(57.5%)	0.0001 **
Phenicols	Chloramphenicol	3(4.5%)	0(0.0)	63(95.4%)	0.0001 **
Aminoglycosides	Gentamicin	6(9.0%)	0(0.0)	60(90.9%)	0.0001 **
	Amikacin	1(1.5%)	1(1.5%)	64(96.9%)	0.0001 **
	Tobramycin	44(66.6%)	2(3.0%)	20(30.3%)	0.0001 **
Nitrofurant-oins	Nitrofurantoin	4(6.0%)	4(6.0%)	58(87.8%)	0.0001 **
P-value		0.0001 **	0.0084 **	0.0001 **	---
** (P≤0.01).					

OmpK36 gene amplification

K. pneumoniae OmpK36 gene was identified using PCR using a particular primer combination to amplify the gene (*OmpK36* -F and *OmpK36* -R). This primer was used to amplify 50 *K. pneumoniae* isolates, Figure (3.6) shows that the PCR product was around 239bp in size.

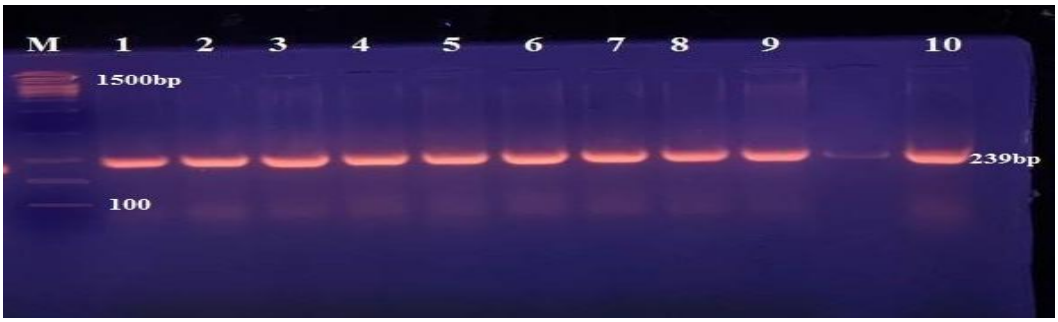

Figure (3.6): PCR product analysis for the OmpK36 gene in *K.pneumoniae* isolates on a 2 % agarose gel electrophoresis and run with a 70 volt.current for 1hrs. M (100-1500bp).Positive K.pneumoniae isolates from lanes (1-10) at 239bp product size.

OmpK35 and *OmpK36* are the major outer membrane porins of *Klebsiella pneumoniae*. These genes play a major role in the passage of antibiotics into the cell because antimicrobial drugs must penetrate the outer membrane first to reach the periplasm, and with β-lactams that are generally hydrophilic and charged, porin channels appear to be the principal route of penetration. Expression of Porins in clinical isolates *Klebsiella pneumoniae* can be altered by factors such as point mutations or insertional interruptions in the coding sequences or the promoter region and the strains lacking both *OmpK35* and *OmpK36* show high levels of antibiotic resistance (16). The mutations also affect conductance, although there is no strict correlation between an apparent increase in pore size due to removal of a huge side chain and increase conductance.

Mutation screening

There are many types of mutations in the ompk36 gene, explained in the table (3) below. Y, Tyrosine; N, Asparagine, R, Arginine ; G, Glycine ; S, Serine ; P, Proline ; V, Valine ; D, Aspartic acid; Q, Glutamine ; A, Adenine; C, Cytosine; G, Guanine; T, Thymine; U, Uracil.

N. of Sample	Sample source	Type of antibiotic	Wild type of codon	Mutant type of codon	Position of codon	Change in amino acid	Position in whole genome	Type of mutation	Name of mutation
4 R	Burn	AMP, MRP, AML.	AAC	AGC	197	N>S	1590563	Substitution	197N>S
			GAC	-A-	199	Frameshift	1590569 1590570	Deletion	Del 199 A
14 R	Urine	AMP,MRP,CFM, RL,CAZ, CRO, TM, CTX.	GAC	G-C	101	Frameshift	2210838	Deletion	Del 101 A
22 R	Urine	AMP, NA, PRL, CIP, CAZ, TM, CN.	GAC	AAC	109	D >N	1590595	Substitution	109 D >N
49 R	Urine	AMP,CFM,PRL, CAZ, CRO, TM, CTX.	GCC	GCT	195	A>G	1590554	Substitution	195A>G
			AAA	AA-	195	Frameshift	1590555	Deletion	Del 195 A
			CAG	-AG	196	Frameshift	159058	Deletion	Del 196 C
11 R	Urine	AMP, MRP, NA, PRL, CIP,	TGA	CTGA	201	Frameshift	2210836	Insertion	Ins 201 C
			TCG	ATCG	203	Frameshift	2210846	Insertion	Ins 203 A
			TCT	ACTC	205	Frameshift	2210854	Insertion	Ins 205

		AML, CAZ, CRO, TM, CTX, CN.		T					AC
13R	Urine	AMP,C, MRP,PRL,AML,CAZ, CRO,TM, CTX.	AAC	GAC	197	N>D	1590561	Substitution	197N>D
17 R	Sputum	AMP,MRP,CFM, NA, PRL,CIP, AML,CAZ,CRO, CTX,F.	CAA = Q	CAG = Q	259	Silent mutation	2864729	Substitution	259 Silent mutation
			TAC	AAC	260	Y>N	2864730	Substitution	260Y>N
22 R	Urine	AMP, NA, PRL, CIP, CAZ, TM, CN.	GAC	AAC	208	D>N	1590596	Substitution	208D>N
41 R	Urine	AMP,CFM,PRL, CAZ,CRO,TM,	-	-	-	-	-	-	
44 R	Urine	AMP,CFM,PRL, CAZ,CRO,TM, CTX	-	-	-	-	-	-	
45 R	Urine	AMP,CFM,PRL, CAZ,CRO,TM, CTX.	-	-	-	-	-	-	
49 R	Urine	AMP,CFM,PRL, CAZ,CRO,TM, CTX.	GCC	GGC	293	A >G	1590550	Substitution	293A >G
			AAA	AA-	195	Frameshift	1590555	Deletion	Del 195 A
			GAC	AAC	238	D>N	1590684	Substitution	238D>N
			-CG	ACG	239	Frameshift	1590689	Insertion	Ins 239 A

Mutation is a very important concept in biology today that leads to differences in genes. A mutation is a permanent change in the sequence of the nitrogenous bases of DNA molecule. Mutation in bacteria has some results such as missense, nonsense, silent, frameshift, and others. Identifying these mutations requires detection methods. Classical methods

such as DNA sequencing is the method highlighted in this study. Many different DNA mutations can occur from them frameshift mutations that includes addition or deletion of base pairs causing a shift in the "reading frame" of the gene. This causes a reading frame shift and all of the codons and all of the amino acids after that mutation are usually wrong. Since the addition of amino acids to the protein chain is determined by the three base codons, when the overall sequence of the gene is altered, the amino acid sequence may be altered as well (17).

The results showed the presence of several mutations in *ompk36* as shown in table (3), where the effect of the mutation on the porin was to change the shape of the porin to prevent the entry of the antibiotic, hence the spread of high drug resistance that has become a serious health problem worldwide and is a major clinical concern. Previous studies showed that inactivation of *ompK36* by insertion-duplication mutagenesis can reduce virulence in a murine peritonitis model of malignant *K.pneumoniae* infection, used in-frame deletion mutagenesis to generate the Δ*ompK35*, Δ*ompK36*, and double-deletion mutants (18). Loss of either *OmpK35* or *OmpK36* porin can lower the permeability of drug into the cell and cause antimicrobial resistance. Loss of *OmpK35* porin can also serve as the first-step mutation for developing resistance to fluoroquinolones in *K. pneumoniae* (19). Particular worry is the resistance to third generation cephalosporins, Carbapenems since β-lactam antibiotics are among the most commonly prescribed drugs, and there are very limited options beyond these drugs with a comparable safety profile and clinical efficacy. Hydrophilic compounds like β-lactam drugs do not readily cross through the hydrophobic lipid bilayer of Gram-negative outer membrane and the presence of porin channels across the outer membrane allow efficient penetration of these antibiotics into the bacterial cell (20).

Conclusions

Klebsiella pneumoniae was identified from urine samples more frequently than other samples isolated from burns and sputum. *K.pneumoniae* is an important cause of urinary tract infections, acute respiratory infections and burns. There is a high prevalence resistance to third and fourth generation of cephalosporins among *Klebsiella pneumoniae* isolates. Mutations that occur in the outer membrane porin *(ompk36*) lead to modulations in these porins and thus prevent the entry of antibiotics to reach their target. The frameshift mutation are more frequent of mutations in *ompk36* and *Klebsiella pneumoniae* isolates that contain the frameshift mutation showed more resistance than others. The antibiotics (Chloramphenicol and Amikacin) are the most sensitive antibiotic to which all isolates have appeared (4.5% and 1.5%) respectively. Statistically there was highly significant difference ($P \leq 0.01$) in the Susceptibility to antibiotics of *K. pneumoniae* infection. Excessive use of over-the-counter treatments leads to bacterial resistance to these drugs, as was the case with ampicillin (AMP), where the resistance to it was 100%. For *K. pneumoniae*, polymerase chain reaction (PCR) is regarded a reliable, reasonably fast, cost-effective, and simple to apply and repeatable method.

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