Mutation in Ompk35 are Evolution Pathway in Antibiotic Resistance

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Abstract
OmpK35 is the major outer membrane porins of Klebsiella pneumoniae. This study was amid to determine the effect of mutation in Ompk35 in antibiotic resistance. This cross-sectional study was, performed on 250 clinical specimens collected from three major hospitals in wasit between. The presence of antibiotic resistance determinants was, investigated by polymerase chain reaction (PCR) method. The purified PCR products of ompk35 positive isolates were, sequenced to screen for mutations in porins. Among the types of mutations that were found in resistant isolates were the frameshift mutations, the mutations that occurred in the ompk35 gene, they are as follows:(78 G>R, 86 S>P, 38 V>D ,40 V>D, Ins 56 CCTATG, Ins 41 T, Ins97 A3, del42 G, del 43 A, del 41 C and del 42 A)
Keyword: Ompk53 and porin

1. 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 screening of mutations that occur in the outer membrane of K. pneumoniae (ompk35 genes)

2. Materials and Methods

2.1. 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.

2.2. 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 .

2.3. Detection of OMPK35gene

OMPK35 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 contains 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 tem-plate.

2.4. 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 sequences of this primer were: Forward, primer:5'ACGGCAACAAACTGGGACTTC3'; Reverse, primer:5'-AGACGGGTTTTTGTGGTCTG-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.

2.5. 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.

2.6. PCR Products Sequencing

The 25-µl PCR products of resist bacteria (58) samples of the analyzed ompk35 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).

2.7. 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.

3. Results and Discussions

3.1. Characteristics of clinical samples

From November 2021 to February 2022, 66 (26.4%) *K. pneumoniae* 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)

Table (1): Clinical samples source and numbers

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

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).					

3.2. OmpK35 gene amplification

Klebsiella pneumoniae OmpK35 gene was identified using PCR using a particular primer combination to amplify the gene (OmpK35 -F and OmpK35 -R). This primer was used to amplify 50 K. pneumoniae isolates, Figure (1) shows that the PCR product was around 208bp in size.

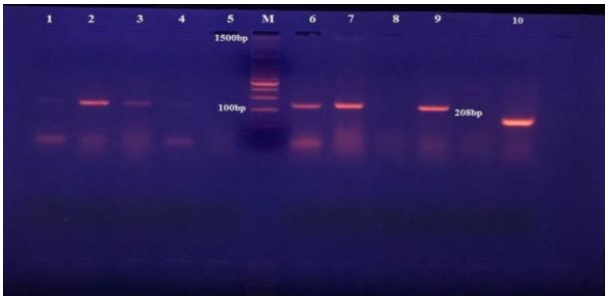

Figure (1): PCR product analysis for the OmpK35 gene in K.pneumoniae isolates on a 2% agarose gel electrophoresis and run with a 70 volt. Current for 1hrs. M (100-1500bp) and Line 1, 2, 3,6,7,9 Positive at 208bp product size... Lane 10 Positive controls and Lane 4, 8 are negative for OmpK35 gene

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.

a. Mutation screening

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 table (3) showed there are many types of mutation in ompk35 gene. the frameshift mutation (insertion and deletion) the more frequent of mutation. R, Arginine; G, Glycine; S, Serine; P, Proline; V, Valine; D, Aspartic acid . 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
11 R	Urine	AMP, MRP, NA, PRL, CIP, AML, CAZ, CRO, M, CTX, N.	CAG	CGG	78	G>R	77280	Substitution	78 G>R
			TCC	CCC	86	S>P	77183	Substitution	86 S>P
14 R	Urine	AMP, MRP, CFM, PRL, CAZ, CRO, TM, CTX	GTC	GAC	38	V>D	3390340	Substitution	38 V>D
17 R	Sputum	AMP, MRP, CFM, NA, CIP, AML, CAZ, CRO, CTX, F.	-GGC	TGGC	41	Frameshift	3390342	Insertion	Ins 41 T
			GTC	-TC	42	Revert mutation	3390350	Deletion	Del 42 G
			------	CCTATG	56	Frameshift	3390384	Insertion	Ins 56 CCTATG
22 R	Urine	AMP, NA, PRL, CIP, CAZ, TM, CN.	-GGT	TGGT	41	Frameshift	3390341	Insertion	Ins 41 T
			GAG	G-G	43	Revert mutation	3390344	Deletion	Del 43A
41 R	Urine	AMP, CFM, CAZ, CRO, TMCTX.	GTC	GAC	40	V>D	3390340	Substitution	40 V>D
			CA-	CAC	41	Frameshift	3390351	Deletion	Del 41C
44 R	Urine	AMP, CFM, PRL, CAZ, CRO, TM, CTX.	-	-	-	-	-	-	
45 R	Urine	AMP, CFM, CAZ, CRO, TM, CTX.	C-C	CAC	42	Frameshift	3390349	Deletion	Del 42 A
49 R	Urine	AMP, CFM, NA, PRL, CIP, CAZ, TM, CTX.	-CGT	ACGT	97	Frameshift	281123	Insertion	Ins 97A

The results showed the presence of several mutations in *ompk35* 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. Single deletion of *ompK35* (Δ*ompK35*) had no significant effect (P-value: 1.00 NS) (Table 4), as The results showed that *K.pneumoniae* isolated from urine had highly significant mutations (P≤0.01) and therefore a high rate of resistance to antibiotics, especially beta-lactams, and the reason for this was due to the frequent use of these treatments.,
Although loss of *OmpK35* appears to be reported more often than OmpK36, separating and identifying these two porin species is often difficult. Based on sequence similarities between the *ompK35* and *ompK36* genes, *OmpK35* is a homolog of *OmpF* (18), and the defective OmpF mutant was resistant to several antibiotics including β-lactams, indicating that OmpF functions as a major component and pathway for the outer membrane penetration of many antibiotics. (19). the absence of *OmpK35* may be one of the contributing factors to the antibiotic resistance of *K.pneumoniae*, and Palasubramaniam *et al.*, 2009(20) have supported this hypothesis.

4. Conclusions

K.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. 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 (ompk35) 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 ompk35 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≤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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