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Molecular Detection of Some Virulence Factors Genes among Gentamicin-Resistant *Klebsiella Pneumoniae* Isolates

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Abstract

Klebsiella pneumoniae (K. pneumoniae) is an important gram-negative pathogen, frequently associated with nosocomially acquired infections that range from mild urinary tract infection to severe bacteremia. So, this study aimed to investigate serotype genes (K1/K2), biofilm gene (pml) and efflux pump gene (ACrAB) among gentamicin-resistant K. pneumoniae isolates. The results showed that out of 481 specimens, 270 (56.13%) showed bacterial growth versus 211 (43.87%) showed no bacterial growth, the ratio between female and male was as follows, 299(62.1%) female and 182(37.9%) male. According Vitek-2 system recorded 94 isolates as K. pneumoniae. Data demonstrated that 42(44.68%) K. pneumoniae isolates were resistant to gentamicin compared with 24(25.53%) and 28(29.78%) of isolates were intermediate and sensitive to this antibiotic respectively. Results of antibiotic susceptibility showed that the highest bacterial resistance was ceftazidime 42(100%), while meropenem 11(26.3%) had the least resistance. PCR amplification results showed that K1 and K2 genes were 5(11.9%) and 35(88.0%) respectively. While ACrAB efflux pump gene showed that of all isolates have this gene 42(100%). Also, PCR results showed that 36 (85.7%) of the isolates had Pml genes.

Keywords: *Klebsiella Pneumoniae*, Drugs Resistance Genes, Virulence Factors Genes

Introduction

Klebsiella pneumoniae constitutes one of the notable *Enterobacteriaceae* family members recognized to be responsible for pneumonia, infection of the urinary tract (UTI), meningitis, sepsis, infection of soft-tissue, and pyogenic liver abscesses (PLA) [1].

Klebsiella pneumoniae is divided into two types: classical K. pneumoniae (cKP) is mainly found within healthcare and long-term care facilities, whereas hyper-virulent K. pneumoniae (hv KP) causes life-threatening illness and organ dysfunction in young, durable individuals from the general population [2]. *K. pneumoniae* constitutes a Gram-negative, unable-to-move, opportunistic bacterium that causes approximately 10% of nosocomial bacterial infections [3]. Due to the existence of numerous virulence genes encoding virulence factors, *K. pneumoniae* is able to attack the immune systems of mammals and trigger a variety of diseases. Biofilm development, hypermucoviscosity, capsule synthesizing, adhesions, iron uptaking, and lipopolysaccharides formation are some of these virulence factors [4, 5, 6]. K. pneumoniae has the ability to form biofilms, which consist of aggregates of bacterial cells that adhere to one another and/or a surface and embed within an extracellular polymeric substance (EPS) such as DNA, polysaccharides, and proteins make up EPS [7, 8].

Materials and Methods

Collection of the Specimens

Present study was involved 481 clinical specimens were randomly collected from patients suffering different infection included burn, wound, diabetic food ulcer and urinary tract infection (UTIs). All were admitted to main health institutes in Al-Najaf City, including Central Public Health Laboratory, Burn Center, and Al-Sadr Medical City as well as some chief clinical laboratories during three months from September to December 2022. The consent of all patients was obtained for sample collection.

Isolates and Bacterial Diagnosis

All specimens involved diabetic foot ulcer swab, burn swab, wound swab as well as urine were collected, the urine specimens collected using sterile containers, midstream urine, after cleaning the genitals from patients with urinary tract infection, and

centrifuged at 2000 rpm for two minutes directly, the sediment was incubated with a brain heart infusion broth at 37 ˚C overnight and streaked on Blood agar, and MacConkey agar surfaces then cubated aerobically in 37 ˚C for overnight. At the same time, the other specimens from the different sources applied same condition of culture media [9]. The final identification was performed using the automated Vitek-2 compact system using ID-GN cards.

Detection of Gentamicin Resistance among K. Pneumoniae Isolates and Antimicrobial Susceptibility Testing

All isolates of K.peumoniae firstly were tested against gentamycin antibiotic using gentamicin disk (10µg) (Bioanalyse,Turkey) and employed on sterile media of Mueller Hinton agar (England) The suspension of all tested isolates were achieved based on 0.5 McFarland standard. However, only gentamicin-resistant K. pneumoniae isolates were tested on several antibiotics included Amikacin (AK, 10 µg), streptomycin (S, 25 µg), Azithromycin (AZM, 15 µg), Doxycycline (DO, 10 µg), Ceftazidime (CAZ, 30 µg), Meropenem (MEM, 10 µg), Levofloxacin (LEV, 5 µg), Norfloxacin (NOR, 30 µg), Ofloxacin (OFX, 5 µg) and Nitrofurantion (F, 300 µg) (Bioanalyse, Turkey), using the disc diffusion technique according to Kirby-Bauer methods [10] and calculate inhibition zone based on the instructions of the Clinical and Laboratory Standards Institute (CLSI) [11].

DNA Extraction and PCR Assay

The instructions provided by a manufacturing company, a genomic DNA extraction micro kit (Favorgen, South Korea) was used to collect all of the nucleic acid for 42 clinical isolates of *K. pneumoniae*. This was done in accordance with the manufacturer's protocol. After ensuring the integrity of the whole DNA sample by storing it in a deep freezer set to -20 degrees Celsius, a PCR analysis was carried out in order to test for the genes listed in Table 1. The equipment for gel documentation was employed for the migration of PCR amplification (bands) at 1% agarose, and then the bands were dyed with ethidium bromide at a concentration of 0.5 g/ml [12].

Table 1: Primer Sequence and condition

Primer target	Sequence (5’ to 3’)	Product size	Annealing (ºC)	Reference
K1-F	GGTGCTCTTTACATCATTGC	1283	54ºC	[13]
K1-R	GCAATGGCCATTTGCGTTAG			
K2-F	GACCCGATATTCATACTTGACAGAG	641	58ºC	[13]
K2-R	CCTGAAGTAAAATCGTAAATAGATGGC			
ACrAB-F	ATCAGCGGCCGGATTGGTAAA	312	53ºC	[14]
ACrAB-R	CGGGTTCGGGAAAATAGCGCG			
Pml-F	GGATCATCTATAATGAAACTG	563	40 ºC	[15]
Pml-R	CTGATAATCAACTTGGAAGTT			

F, forward; R, reverse

Results and Discussion

Patients and Bacterial Growth

Results of this study showed among 481 patients was 270 (56.13%) bacterial growth compared with 211 (43.86%) no bacterial growth. the results of biochemical tests, Vitek-2 system showed among 481specimens, when collecting specimens, the ratio between men and women was as follows, in wounds 33 (58.9%) males and 23(41.1%) females, diabetic foot 37(68.51%) males and 17(31.49%) females and burns 25(56.81%) males and 19(43.19%) females, while the percentage of females in urinary tract infection was higher than that of males 87(26.60%) while the percentage of females was (73.40%). Fig 1 current study recorded 94(19.54%) were obtained from 481 specimens. The results showed that K. pneumoniae. 16 (17.02%) from wounds, 15 (15.96%) from burns, 18 (19.15%) from diabetic foot and 45 (47.87%) from urine, as shown below in Table (2).

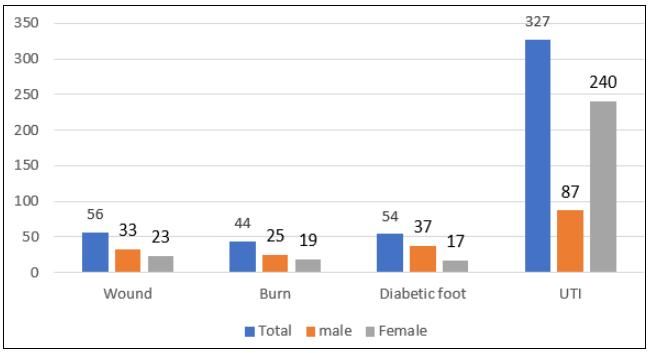

Fig 1: Distribution of specimens according to Gender

Table 2: Specimens collected from Different source

S. No	Source	Diabetic foot		UTI		Burn		Wound		Total
	Gender	M	F	M	F	M	F	M	F	
1	No growth	7	4	46	115	11	9	10	9	211(43.86)
2	klebsiella pneumoniae	12	6	16	29	8	7	10	6	94(19.54%)
3	Other G-	18	7	25	96	6	3	13	8	176(36.59)
4	Total	54(11.22%)		327(67.98%)		44(9.14%)		56(11.64%)		481(100%)

M, Male; F, Female

This result is comparable to that measured by Naqid *et al.* [16] in Iraq, they observed that the evaluation of 130 positive K. pneumoniae cultures from diverse clinical samples. The urine samples yielded the greatest number of K. pneumoniae isolates (n = 86; 66.2%), followed by blood samples (n = 16; 12.3%) and wound biopsies (n = 13; 10%). Ali and Ismail

[17] obtained 88 (29.33%) Klebsiella isolates from 300 distinct clinical specimens in Erbil Province in their experiment. Okwuonu and Chukwura [18] obtained 80 (40%) Klebsiella isolates from 200 distinct clinical specimens in Nigeria in their experiment.

Numerous studies are conducted by researchers in Iraq, such as obtaining 61 Klebsiella isolates (33.88%) from a total of 180 samples collected from two clinical sources in Baghdad province [19] In a local study by Hasan *et al*., [20] discovered that out of a total of 207 Gram-negative bacteria isolates, K. pneumoniae was the second most prevalent with a prevalence rate of 35.74 %; based on gender, 47 (63.51%) of the K. pneumoniae isolates were females and 27 (36.49%) were males.

Antibiotic Susceptibility of Gentamicin-Resistance K. Pneumoniae

Results showed from among 94 (100%) K. pneumoniae isolates found 42(44.86%) K. pneumoniae isolates were resistant to gentamicin, while 24(25.53%) and 28(29.78%) of isolates were intermediate and sensitive to this antibiotic respectively (see Fig 2).

There were several previous studied showed elevation of gentamicin resistances among K. pneumoniae, however, Chiemchaisri, *et al*., [21] they found that resistance of K. pneumoniae to gentamicin was 100% in Thailand. While Safika *et al*., [22] they found that resistance of K. pneumoniae to gentamicin was 45.0% in Indonesia. The high rate of resistance among of K. pneumoniae had concern in the country, therefore require used new strategies to decrease level of resistance.

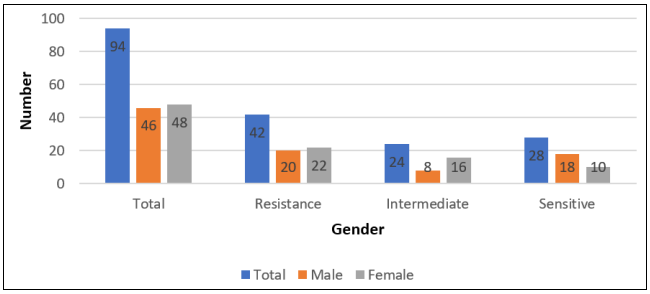

Fig 2: *K. Pneumoniae* isolates classified according to gentamicin Susceptibility and sex

Antimicrobial Susceptibility Testing of Gentamicin-Resistant K. Pneumoniae Isolates

The result in Table 3 showed ceftazidime at rate 42 (100%), This result was in accordance with a pervious study in Baghdad by Murtadha [23] who found that resistance of K. pneumoniae isolates to Ceftazidime was 96.1%. the rate of resistance among the isolates decreased to 11 (26.3%) toward meropenem drug which represent member of the carbapenem subclass compared with 9(21.4%) and 22(52.3%) were intermediate and sensitive respectively. The level of resistance of K. pneumoniae isolates to azithromycin (macrolide class) in this study was 21(50%) respectively. This result was in accordance with a pervious study in Baghdad by Ruqaia Ali [24] who found that resistance of k. pneumoniae isolates to azithromycin was (56.0%). The data of antibiotics susceptibility belonging to the aminoglycoside class showed results 17 (40.5%) of isolates was resistance amikacin, while 19 (45.2%) of isolates were resistance to streptomycin. For Tetracycline antibiotics class, the study involved of Doxycycline antibiotic, where the resistance rate was 18 (42.8%). The results also showed different resistance to the class of fluoroquinolones antibiotics, as the study included three antibiotics belonging to this class, and the resistance rate was as follows 26 (62.7%), 17 (40.5%) and 16 (38%) of isolates was resistant to Levofloxacin, Norfloxacin and Ofloxacin respectively. The study also included the class of nitrofuran antibiotics, the percentage of resistance of the isolates was 37 (88.2%) toward Nitrofurantoin antibiotic while 2(4.7%) and 3(7.1%) were intermediate and sensitive respectively. and this result is similar to the result obtained by Khaled Ibrahim [25] in Ramadi who found the percentage of resistance to Nitrofurantoin was (85%).

Table 3: Antibacterial agent susceptibility of gentamicin-resistant *K. pneumoniae* isolates

Antimicrobial Agents	Resistance N (%)	Intermediate N (%)	Sensitive N (%)
Amikacin	17 (40.5%)	4 (9.5%)	21 (50%)
Streptomycin	19 (45.2)	11 (26.1%)	12 (28.7%)
Azithromycin	21 (50%)	0	21 (50%)
Doxycycline	18 (42.8%)	5 (11.9%)	19 (45.3%)
Ceftazidime	42 (100%)	0	0
Meropenem	11 (26.3%)	9 (21.4%)	22 (52.3%)
Levofloxacin	26 (62.7%)	15 (35%)	1 (2.3%)
Norfloxacin	17 (40.5%)	5 (11.9%)	20 (47.6%)
Ofloxacin	16 (38%)	3 (7.1%)	23 (54.9%)
Nitrofurantoin	37 (88.2%)	2 (4.7%)	3 (7.1%)

Molecular Detection of K1 and K2 Genes in Clinical of Klebsiella Pneumoniae Isolates

The molecular results of the current research showed that 5 (11.9%) of Klebsiella pneumoniae isolates were harbored positive bands at correct position for K1. At same respect, data on PCR showed 35 (88.0%) K2 isolates were positive for genes, as shown in the respectively Fig 3 and Fig 4. There previous studies done about detected these genes, however a study achieved in Iraq done by Qasim and Khalid [26] discovered that the k1 gene was present in 12% of isolates, which is congruent with the result obtained in this research. However, their result for the k2 gene differs from the current study's result of 8%. El-Ashry *et al*., [27], who conducted a study in Egypt, discovered that K1 was prevalent in 40/87 (45.9%) of isolates, while 18/87 (20.6%) had been categorized as K2 capsular type.

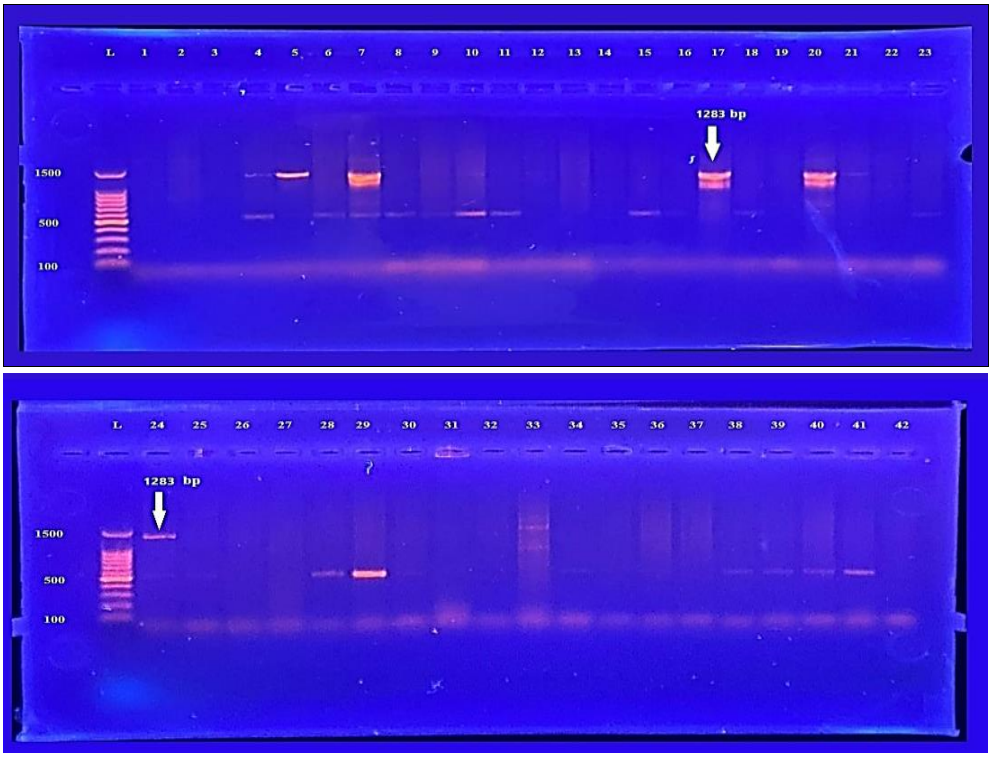

Fig 3: PCR amplification of K1 gene among 42 gentamicin-resistant *K. pneumoniae* isolates

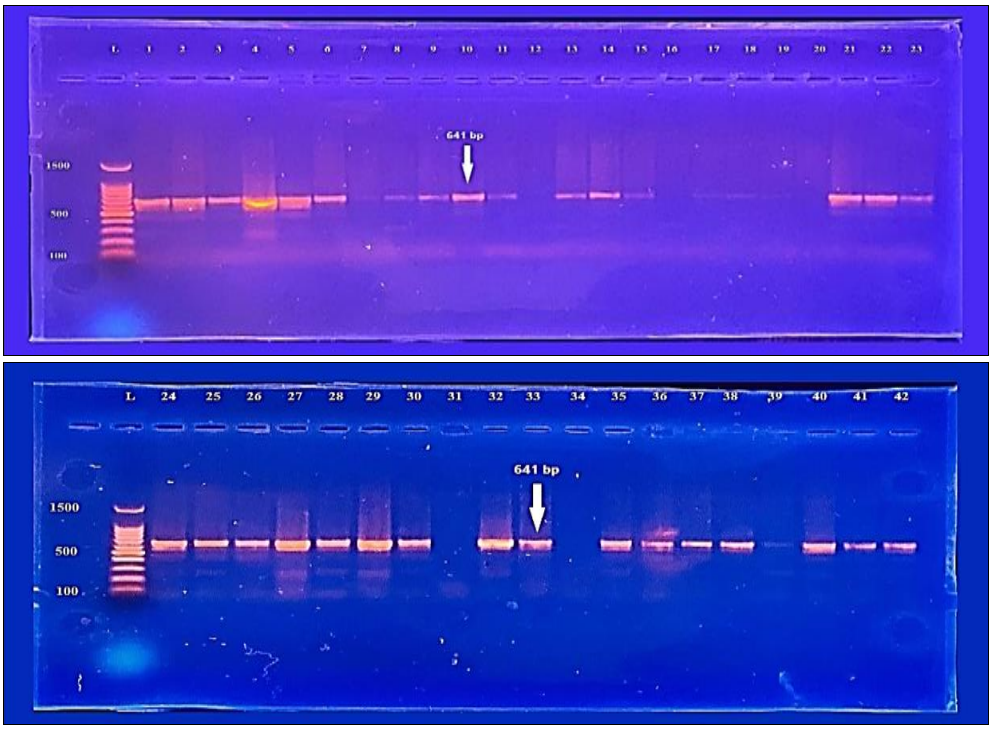

Fig 4: PCR amplification of K2 gene among 42 gentamicin-resistant *K. pneumoniae* isolates

Molecular Detection of ACrAB Efflux Pump Genes in Clinical of *Klebsiella Pneumoniae* Isolates

The efflux pumps gene of ACrAB plays a crucial role in resistance to antibiotics in multidrug-resistant K. pneumoniae isolates such as ciprofloxacin and other fluoroquinolones, thereby decreasing the intracellular concentration of the antimicrobial agents [28, 29]. To investigate the frequency of the ACrAB efflux pump gene among 42 isolates of gentamicin-resistant K. pneumoniae through using of specific primers in PCR technology. according to the results of this investigation, the distribution of the AcrAb efflux gene was 100% (42/42) as shown in Fig 5. In accordance with a previous study conducted by Abid, [30] in AL-Diwaniyah, the result of ACrAB efflux pumps was 100 percent. Other research by Alsanie, [31] in Saudi Arabia determined that the prevalence of ACrAB efflux pumps was 100% (23/23). However, one of the reasons for the phenotypic resistance to antibiotics among gentamicin-resistant K. pneumoniae isolates in the current study may be due to the prevalence of this gene.

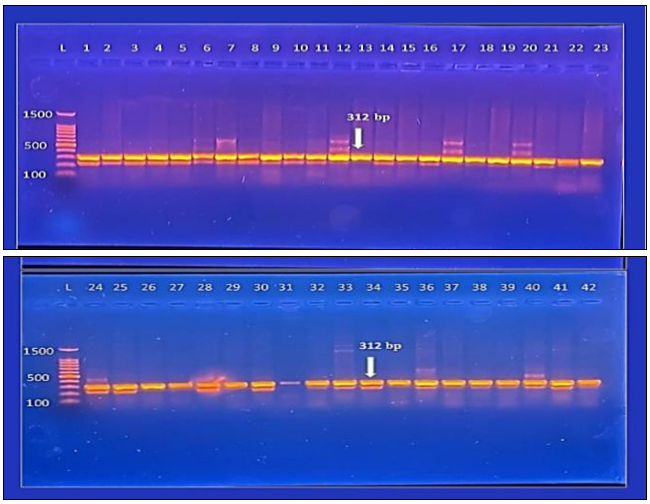

Fig 5: PCR amplification of ACrAB gene among 42 gentamicin-resistant *K. pneumoniae* isolates

Molecular Detection of PM1 Gene in Clinical of Klebsiella Pneumoniae Isolates

For many microorganisms, including K. pneumoniae, having the capacity to generate biofilms is an essential virulence trait. Gram-negative, encapsulated bacteria frequently linked to nosocomial infections. It has been estimated that 65 to 80 percent of infections caused by bacteria are related to biofilm [32]. the molecular detection of pm1 gene by using specific primer for Klebsiella Pneumoniae isolates revealed positive amplification for 36(85.7) isolates as shown in Fig 6. This result was consistent with the findings of Allawi and Motaweq [33], who found that 100% of clinical specimens contained the pml gene and this clinical specimen demonstrated significant biofilm formation. The presence of this gene among gentamicin-resistant Klebsiella pneumoniae isolates is an important factor in virulence, as it has a major role in antibiotic resistance as well as its role in preserving the bacteria and staying as long as possible in resistance to difficult conditions.

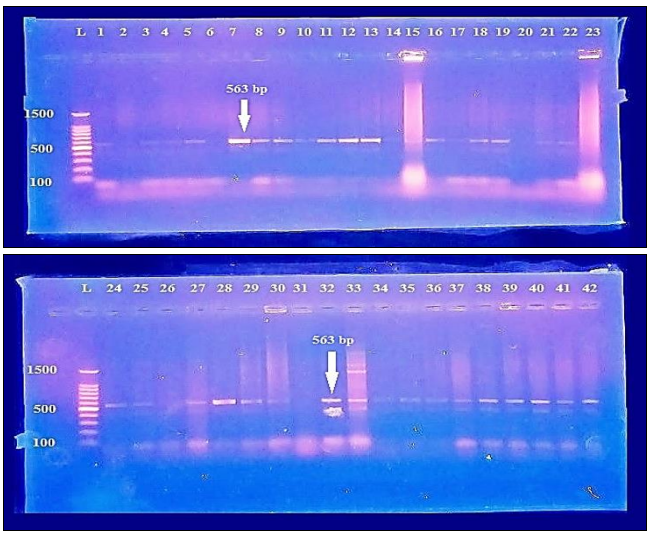

Fig 6: PCR amplification of Pml gene among 42 gentamicin-resistant *K. pneumoniae* isolates

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