Genetic detection of folate pathway inhibitors and outer membrane porins genes among gentamicin-resistant *klebsiella pneumoniae* isolates

Faris Hanoon Ali Shallal[1*] and *Nabil Salim Saaid* Tuwaij[1]

[1]University of Kufa, Department of Biology, Faculty of Science, Kufa, Iraq

Abstract. Infectious diseases are one of the leading causes of morbidity and mortality worldwide, and antibacterial resistance exacerbates the situation. So this study aimed to investigate genes responsible for folate inhibitor pathway antagonists and outer membrane protein genes 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. According vitek-2 system recorded 94 isolates as *K. pneumoniae*. Data demonstrated that 42/94 (44.68%) K. pneumoniae isolates were resistant to gentamicin compared with 24/94(25.53%) and 28/94(29.78%) of isolates were intermediate and sensitive to this antibiotic respectively. Results of antibiotic susceptibility showed that the highest bacterial resistance was piperacillin 41/42 (97.7%). while netilmycin 16/42 (38%) had the least resistance. PCR amplification results showed that dfr-B, Dfr-G and Dfr-K were 13/42 (30.9%), 17/42 (40.4%) and 37/42 (88.0%)) respectively. While Dfr-A did not detect. Also PCR results showed that 39 (92.8%) and 32 (76.1%) of the isolates had Ompk35 and Ompk36 genes respectively.

1 Introduction

Klebsiella pneumoniae (K. pneumoniae) constitutes an encapsulating Gram-negative bacterium which colonizes numerous human body sites, including the gastrointestinal system, tract of respiratory, oral cavity, as well as skin [1]. K. pneumoniae is regarded as one of the most significant opportunistic pathogens related to nosocomial and community-acquired infections, especially in immune-compromised hospitalized patients; infections caused by K. pneumoniae also increase with the indiscriminate use of antimicrobials. K. pneumoniae primarily causes infections in the respiratory and urinary systems[2]. Klebsiella pneumoniae predominantly has caused infectious diseases in immunosuppressed individuals. However, its emergence and spread are spreading even to healthy and immunocompromised people. Furthermore, the K. pneumoniae strain is becoming increasingly antibiotic-resistant, making treatment of infection with the strain extremely difficult [3]. Once infection is

* Corresponding author: farishanoon75@gmail.com

established K. pneumoniae forms a biofilm that enables evasion of the host's defenses [4]. The existence of hyper-virulent clones is a key feature of K. pneumoniae and typically show increased pathogenesis not commonly associated with antimicrobial resistance [5]. Pathogenicity of K. pneumoniae is due to the presence of many virulence genes which encode virulence factors that allow it to attack the immune system of mammalians and cause many kind of diseases. Some of these virulence factors are: biofilm formation, hypermucoviscosity, capsule synthesis, adhesions, iron uptake and lipopolysaccharides formation [6,7,8]

2 Materials and methods

2.1 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.

2.2 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.

2.3 Detection of gentamicin resistance among K. pneumonae 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 aztreonam (ATM, 30 µg), ciprofloxacin (CIP, 10 µg), amoxicillin /clavulanate (AMC, 30 mcg), cefpodoxime (CPD, 30 µg), trimethoprim sulfamethoxazole (STX, 25 µg), Netilmicin (NET, 30 µg), tetracycline (TE, 10 µg), Tobramycin (TOB, 10 µg), Cefepime (FEP, 30 µg) and Piperacillin (PRL, 100 µg)(Bioanalyse, Turkey), using the disc diffusion technique according to the instructions of the Clinical and Laboratory Standards Institute (CLSI) [10].

2.4 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. pneumonaiae. 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 thereafter (11).

Table 1. Primers and condition used in the study

Primer target	Sequence (5' to 3')	Product size	Annealing (ºC)	Reference
DfrA-F	CACTTGTAATGGCACGGAAA	270	57	12
DfrA-R	CGAATGTGTATGGTGGAAAG			
dfrB-F	AATTGTGTTAAATTAAAGATAACTT	572	43 ºC	12
dfrB-R	TAAGTATTCTTTAGATAAATCGGAT			
dfrK-F	GCTGCGATGGATAATGAACAG	321	49 ºC	12
dfrK-R	GGACGATTTCACAACCATTAAAGC			
dfrG-F	TGCTGCGATGGATAAGAA	405	57 ºC	12
DfrG-R	TGGGCAAATACCTCATTCC			
Ompk35-F	CTCCAGCTCTAACCGTAGCG	241	43 ºC	13
Ompk35-R	GGTCTGTACGTAGCCGATGG			
Ompk36-F	GAAATTTATAACAAAGACGGC	305	51ºC	13
Ompk36-R	GACGTTACGTCGTATACTACG			

3 Result and discussion

3.1 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 and PCR showed among 481specimens, 94isolates were identified as *K.pneumoniae* isolates. K. pneumoniae isolates were mostly observed from urine 45(47.87.4%) while the ratio in wounds 16(17.02%) and 18 (19.14%) for diabetic foot, 15 (15.95%) for burns specimens. However, the distribution of 94 isolates of *K. pneumoniae* in patient according sex and source as show in figure 1.

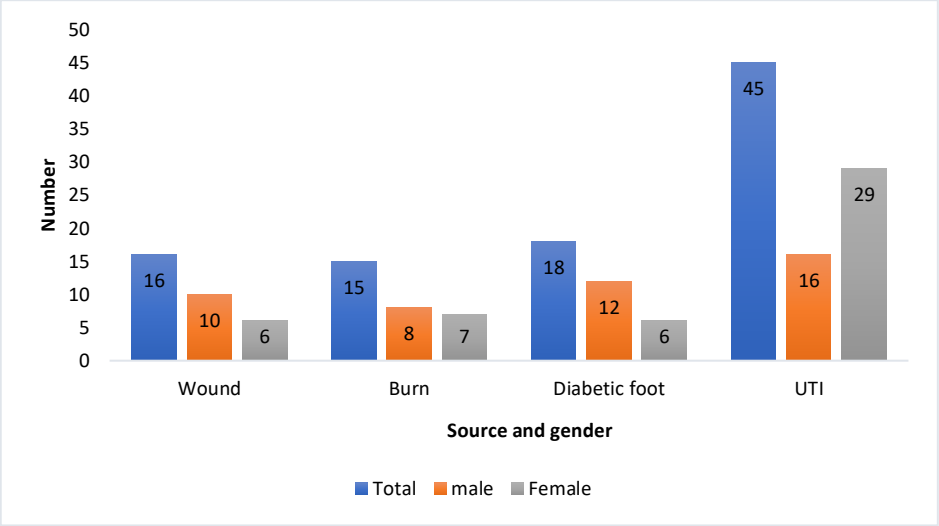

Fig. 1. K. pneumoniae isolates classified according to Source and Gender

This result is comparable to that measured by Naqid *et al.*,[14] 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 [15]obtained 88 (29.33%) Klebsiella isolates from 300 distinct clinical specimens in Erbil Province in their experiment. Okwuonu and Chukwura [16] 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 [17

In a local study by Hasan *et al.*, [18] 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. In a local study by Raouf et al., [19] who found out of a total of 308/511 (60.3%) Gram-negative bacteria isolates, K. pneumoniae was prevalent with the percentage of 163/511 (31.9%), from other hand depending on the gender the *K. pneumoniae* isolates were 61 (37.4%) females and 102 (62.6%) males.

In a local study by Al-Rubaye *et al.*,, [20] they found out of a total of 172 bacteria isolates, *K. pneumoniae* was frequency with the percentage of 33.72%, depending on the gender the K. pneumoniae isolates were 44 (75.86%) in females and 14(24.41%) in males.

3.2 Antibiotic Susceptibility of gentamicin- resistance K. pneumoniae

Results show from among 94 (100%) K. pneumoniae isolate and by using disk diffusion According to Kaurby-baur methods for gentamicin antibiotic demonstrated that only 42(44.86%) K. pneumoniae isolates were resistant to gentamicin, while 24(25.53%) and 2828(29.78%) of isolates were intermediate and sensitive to this antibiotic respectively. As show table 2.

Table 2. Gentamicin Susceptibility of *K. Pneumoniae* isolates

Bacterial name	**Total**		**Resistance**		**Intermediate**		**Sensitive**	
	94(100%)		42(44.86%)		24(25.53%)		28(29.78%)	
K. Pneumoniae	M	F	M	F	M	F	M	F
	46	48	20	22	8	16	18	10

F, female; M, male

There were several previous studied showed elevation of gentamicin resistance among K. pneumonae, 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. pneumonia had concern in the country, therefore require used new strategies to decrease level of resistance.

3.3 Antimicrobial susceptibility testing of gentamicin-resistant K. pneumonae isolates

The result in table 3 showed 41(97.7%) of gentamicin-resistant K. pneumonae isolates were resistance to Piperacillin. similarly study in Baghdad by Murtadha [23] who found that resistance of K. pneumoniae to piperacillin was 100%. while the members of cefepime and cefpodoxime revealed resistance rate 25 (60%) and 34 (81%) respectively. Also 27 (64.7%) of isolates was resistance to aztreonam. The resistance of bacteria toward amoxicillin-clavulanic acid was (67.1%). The results showed that 23(54.7%) and 16 (38.1%) of isolates was resistance tobramycin and netilmicin respectively. Rate of tetracycline resistance was 17 (40.5%). this result is similar to the result obtained by Al-Janaby and Al-Hasnawi [24], they found that 44% of *K. pneumoniae* isolates were resistant to tetracycline, while rate of ciprofloxacin resistance was 29 (69.2%), This result was convergent with a previous study by Ferreira *et al*. [25] in Brazil. This pathogen showed that 32 (76.2%) was resistance to Trimethoprim/ This result is similar to the result obtained by Vo [26] in Vietnam who found that resistance of *K. pneumoniae* isolates to trimethoprim-sulfamethoxazole was (76.47%). The ability of this pathogen to resistance to different classes of drugs may be return the several factors such as muse use of drugs as well as it had several mechanisms of drug resistance like decrease permeability, efflux pump or producing different enzymes cleavage the antibiotics.

Table 3. Antibacterial agent susceptibility of gentamicin-resistanct *K. peumoniae* isolates.

Antimicrobial agent	Resistance	Intermediate	Sensitive
Tobramycin	23 (54.7%)	3 (7.1%)	16 (38.2%)
Netilmicin	16 (38.1%)	5 (11.9%)	21 (50%)
Tetracycline	17 (40.5%)	6 (14.2%)	19 (45.3%)
Piperacillin	41 (97.7%)	1 (2.3%)	0
Amoxicillin-clavulanate	28 (67.1%)	9 (21%)	5 (11.9%)
Cefepime	25 (60%)	8 (19%)	9 (21%)
Cefpodoxime	34 (81%)	1 (2.3%)	7 (16.7%)
Aztreonam	27 (64.7%)	1 (2.3%)	14 (33%)
Ciprofloxacin	29 (69.2%)	7 (16.6%)	6 (14.2%)
Trimethoprim-sulfamethoxazole	32 (76.2%)	0 (0%)	10 (23.8%)

3.4 Detection of folate pathway inhibitors genes among gentamicin-resistance *K. pneumoniae* isolates

Results of PCR showed that Dfr-B, Dfr-G and Dfr-K were 13/42 (30.9%), 17/42 (40.4%), and 37/42 (88.0%) respectively (figure 2 and 3). while Dfr-A gene was absent. A locally study Al-Ghazaly, and Tuwaij, [12], they observed that drf-G and drf-A genes among clinical Acinetobacter spp isolates were. 28 (100%), and 21 (75%) respectively, while drf-B and drf-K genes were not recorded. International study done by Brolund, et al [27] they revealed that dfr-genes observed in K. pneumoniae at rate 69% and the genes dfrA5, dfrA8 and dfrA12 were more common. At same respect, current finding agree with other study that indication that trimethoprim/sulfamethoxazole resistance is caused by the acquiring of mobile genes such as dfr and sul genes [28].

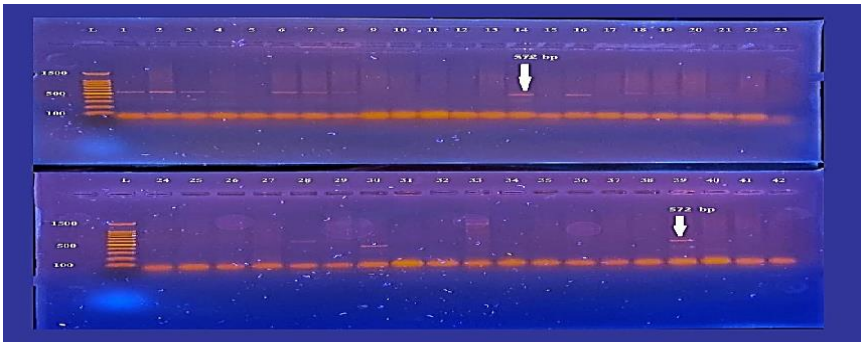

Fig. 2. PCR amplification of Dfr-B gene among 42 gentamicin-resistant *K. pneumoniae* isolates

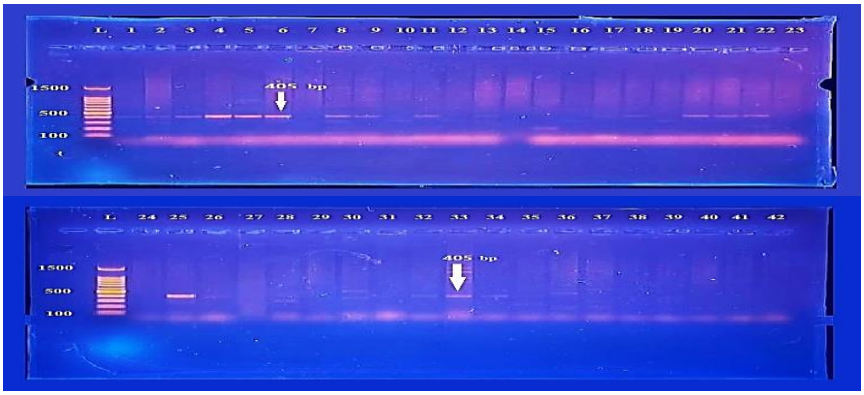

Fig. 3. PCR amplification of Dfr-G gene among 42 gentamicin-resistant K. pneumonia isolates. Detection of Outer Membrane Porins OmpK35 and OmpK36 to multidrug resistance and virulence among K. pneumoniae isolates.

Results showed that OmpK35 and OmpK36 were 39 (92.8%) and 32 (76.1%) respectively (Figure, 4 and 5). In accordance with the findings of a previous investigation conducted in Baghdad by Muhsin et al., [29] they found the results was 51 (85%) of the total 60 isolates had positive results for OmpK35. Current results were high from finding was observed in Saudi Arabia by Ejaz [30] who discovered the Ompk36 result to be 72.5%. However, one of resistance mechanisms in current study exist high rate of OmpK35 and OmpK36 genes among gentamicin-resistance *K. pneumonia*e isolates and this agree with Choi and Lee [31]

they mention that the outer membrane proteins have role in modulate the permeability of cells and resistance to antibiotics.

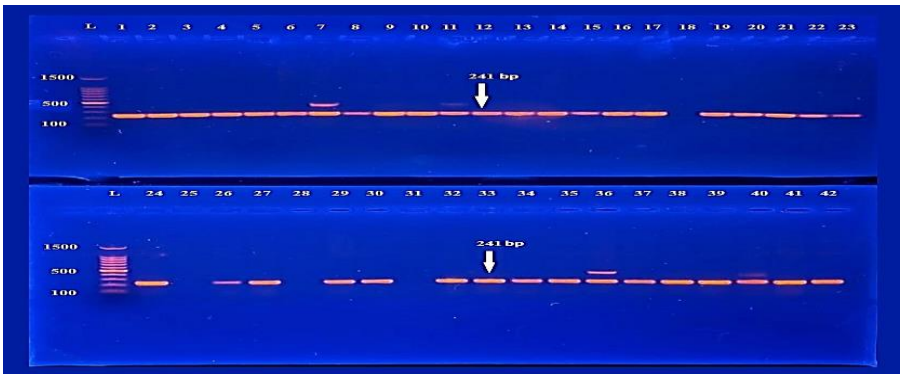

Fig. 4. PCR amplification of Ompk35 gene among 42 gentamicin-resistant K. pneumonia isolates

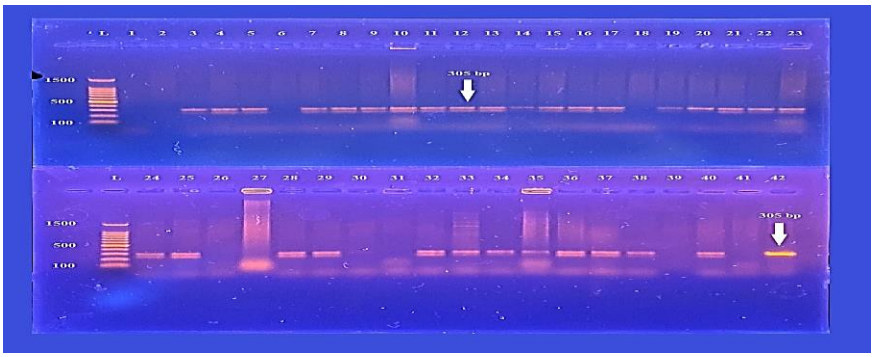

Fig. 5. PCR amplification of Ompk36 gene gene among 42 gentamicin-resistant K. pneumonia isolates

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