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Prevalence of Tetracycline Resistance Genes *tet* (*A, B, C, 39*) in *Klebsiella pneumoniae* Isolated from Tehran, Iran

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ABSTRACT

Background and Aim: *Klebsiella pneumoniae* is one of the three pathogens that has become a global disease control and treatment problem due to its resistance to common antibiotics. For this reason, it is crucial to study the genes that cause antibiotic resistance in it. Therefore, the aim of this study was to investigate the phenotypic and genotypic frequency of tetracycline resistance in clinical isolates of *K. pneumoniae* in Tehran, Iran.

Materials and Methods: In this study, 100 isolates of *K. pneumoniae* isolated from clinical samples (urine) during 2018-2019 were studied. In addition to microbial and biochemical phenotypic tests, genotypic tests were conducted to determine the frequency of antibiotic resistance genes *tet* (*A, B, C, 39*).

Results: Out of 100 isolates of *K. pneumoniae*, 49 isolates were resistant to tetracyclines. The results of multiplex PCR showed that 31 samples were positive for *tetA* gene, 8 isolates for *tetB* gene, 21 samples for *tetC* and, and 8 isolates for tet39. None of the isolates were positive for all four tetracycline genes.

Conclusion: The results of this study showed that the isolates were positive for at least one gene and at most 2 tetracycline resistance genes. The *tetA* gene showed the highest frequency and the lowest frequency was demonstrated by *tetB*. The highest binary combination of genes was *tetA-tetC,* and the lowest was *tetA-tet39*.

Keywords: Antibiotic resistance, *Klebsiella pneumoniae*, Multiplex PCR, Tetracycline

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1 Introduction

Increasing resistance of *Klebsiella pneumoniae* to antibiotics has made it one of the three pathogens threatening global health. *Klebsiella* is responsible for more than 10% of nosocomial infections **(1)**. *K. pneumoniae* is a gram-negative pathogen belonging to the *Enterobacteriaceae* family, which causes several serious infections such as liver abscess, pneumonia, bacteremia and urinary tract infection (UTI) **(2-4)**. *K. pneumoniae* is the most critical nosocomial infection due to its high mortality rate **(5)**.

Klebsiella pneumoniae has two pathotypes, Hypervirulent *K. pneumoniae* (hvkp) and classical *K. pneumoniae* (Ckp), which differ in phenotypic and genotypic chara-cteristics **(6, 7)**. Classical *K. pneumoniae* is the first pathotype to cause most infections. Hyperviolants are a type of *Klebsiella* pathotype with several bioma-rkers, including *peg-344* (virulence of pulmonary infection), iroB, iucA (central nervous system invasion) **(8, 9)**, and *rmpA-rmpA2*, and *macA* (increased prod-uction of antiphagocytic capsules) **(7, 10)**. The Ckp and hvkp

pathotypes are challenging to treat due to their antimicrobial resistance genes **(7)**.

Klebsiella pneumoniae is a Superbug bacterium as it produces Extended-spectrum beta-lactamases (*ESBL*), carbapenemases, mobilized colistin resistance (*mcr-1*), and resistance to a large number of antibiotics (MDR-XDR) **(11, 12)**. These bacteria pose many challenges in treatment **(13, 14)**. Tetracyclines are currently widely used in livestock and humans due to their low toxicity, broad-spectrum activity against beta-lactamase-producing *K. pneumoniae*, tolerability, and easy market access **(15, 16)**.

Unfortunately, the indiscriminate use of these antibiotics has led to antibiotic resistance. Tetracycline resistance is caused by three mechanisms. First, overexpression of efflux pumps AcrAB-TolC and OqxAB, which reduces the cell's permeability to antibiotics due to the performance of efflux pumps and antibiotic resistance. Efflux genes are present in gram-positive and gram-negative bacteria **(17)**. Second, ribosomal protection proteins, which protect ribosomes (S30 and S16) from tetracycline, alter the structure of these proteins, causing resistance to doxycycline and minocycline. Third, enzymatic changes in antibiotics also cause resistance. The *tetX* gene causes antibiotic resistance due to tetracycline enzyme inactivation **(18, 19)**. Currently, 23 genes encode the efflux pump and 11 genes encode it with ribosomal protection proteins **(20, 21)**.

Therefore, due to the development of multidrug resistance to antibiotics in *K. pneumoniae* and various mutation mechanisms, the study of the pattern of antibiotic resistance leads to the appropriate administration of antibiotics and faster recovery of related infections. The aim of this study was to investigate the phenotypic and genotypic resistance of tetracyclines in *K. pneumoniae* isolates in patients with urinary tract infections (UTTI) in Tehran hospitals, Iran.

2.Materials and Methods

Isolation and Identification of Isolates

In this study, 100 isolates of *K. pneumoniae* were isolated from clinical samples (urine) (2018-2019) from a major hospital in Tehran, Iran. Isolates were identified using conventional phenotypic and biochemical methods **(22)**.

Antibiotic Susceptibility Testing

After identifying *K. pneumoniae* and culturing them, the antibiotic resistance pattern of *K. pneumoniae* isolates was performed by standard disk diffusion method (Kirby-Bauer) according to CLSI (2016) instructions. Antibiotic resistance was determined for cefotaxime (CTX, 30g), ceftazidime (CAZ, 30g), erythromycin (E, 15g), and tetracycline (TET, 30g) (Padtan Teb - Isfahan). The results of antibiotic susceptibility testing of the samples after 24 hours of incubation at 37°C according to the standard table were evaluated based on the diameter of the stunted state, and the samples were classified into 3 groups: sensitive (S), semi-sensitive (I) and resistant (R) **(22, 23)**.

DNA Extraction

The bacteria were cultured in LB medium at 37°C. After examining the turbidity of the tubes at 600 nm, bacterial DNA was extracted by a modified boiling method using STET buffer (Tris-Hcl 10 mM, NaCl 0.1 mM, EDTA 1 mM, pH = 8, Triton X -100) (Merck-Germany), the bacteria were extracted. Finally, the quality and quantity of genome concentration were evaluated by spectro-photometer at 260/260 nm. The extracted genome was stored at -20°C **(24)**.

Molecular Study of *tet39*, *tetB*, *tetC* ، *tetA* Genes

After DNA extraction, tetracycline genes were analyzed by multiplex PCR. PCR reaction was performed in a total volume of 25 µL containing 12 µL of Mastermix, 10 µL of distilled water, 0.5 µL of each specific primer (Table 1) (Pishgam Company - Tehran), and 1 µL of sample DNA. PCR conditions for gene amplification were designed based on the binding temperature of the primers (Table 2). Thermocycler (Bio-Rad, USA) was used for PCR reaction. PCR products were analyzed by electrophoresis on 2% agarose gel containing a safe stain (Pishgam - Tehran), and the presence of tetracycline genes in *K. pneumoniae* isolates was determined.

Table 1. The sequence of primers used for multiplex PCR of tetracycline genes, PCR product size, and binding temperature

Primer	Primers' sequence	PCR product size	Connection temperature	Reference
tetAF	GTAATTCTGAGCACTGTCGC	956bp	56 °C	(25)
tetAR	CTGCCTGGACAACATTGCTT	956bp	56 °C	(26)
tetBF	CTCAGTATTCCAAGCCTTTG	415bp	56 °C	(26)
tetBR	ACTCCCCTGAGCTTGAGGGG	415bp	56 °C	(26)
tetCF	CCTCTTGCGGGATATCGTCC	505bp	56 °C	(26)

Primer	Primers' sequence	PCR product size	Connection temperature	Reference
tetCR	GGTTGAAGGCTCTCAAGGGC	505bp	56 °C	(26)
tet39F	CTCCTTCTCTATTGTGGCTA	701bp	56 °C	(26)
tet39R	CACTAATACCTCTGGACATCA	701bp	56 °C	(26)

Table 2. Thermal program used for genes used for PCR

Denaturation	5 min	1 Cycle	95°C
Denaturation	30 s	30 Cycle	95°C
Annealing	30 s	30 Cycle	56°C
Extension	30 s	30 Cycle	72°C
Final extension	5 min	1 Cycle	72°C

3.Results

Out of 100 clinical samples (urine), 54 samples were collected from male subjects and 46 samples from females, and their age range was between 30 and 80. According to the CLSI table (2016), there were 42 tetracycline-resistant samples, 12 cefotaxime-resistant isolates, 15 ceftazidime-resistant isolates, and 83 erythromycin-resistant isolates (Figure 1). According to the PCR multiplex test results, tetracycline resistance is high in *K. pneumoniae* isolates. So that 31 samples were positive for *tetA* gene, 8 samples for *tetB* gene, 21 samples for *tetC* gene, and 8 isolates for *tet39* gene. Also, 5 samples were positive for *tetA* and *tetB* genes, 4 isolates for *tet39* and *tetA* genes, and 14 samples for *tetA* and tetC genes. Most samples were positive for the *tetA* gene. No isolate tested positive for all four genes simultaneously (Figures 1, 2 and 3).

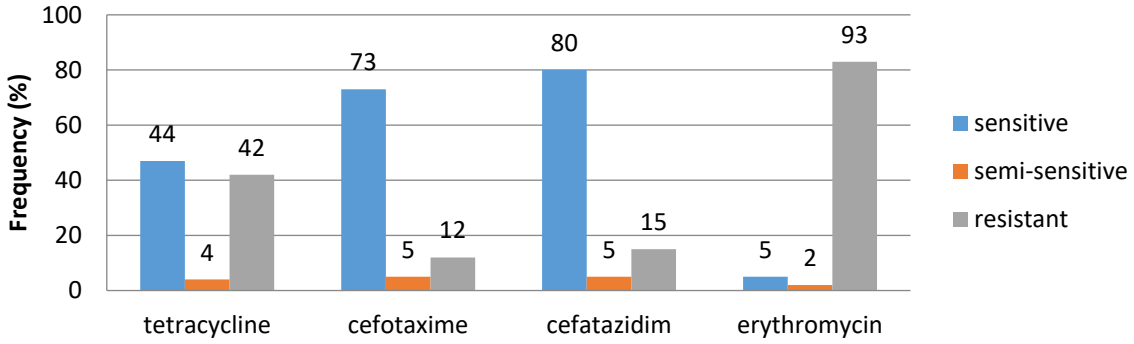

Figure 1. Percentage of antibiotic resistance and susceptibility in *K. pneumoniae*

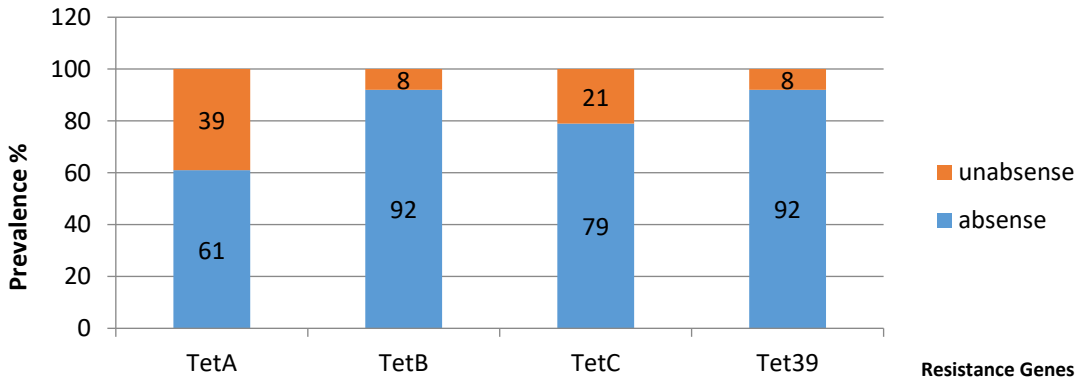

Figure 2. Percentage of tetracycline resistance genes in *K. pneumoniae*

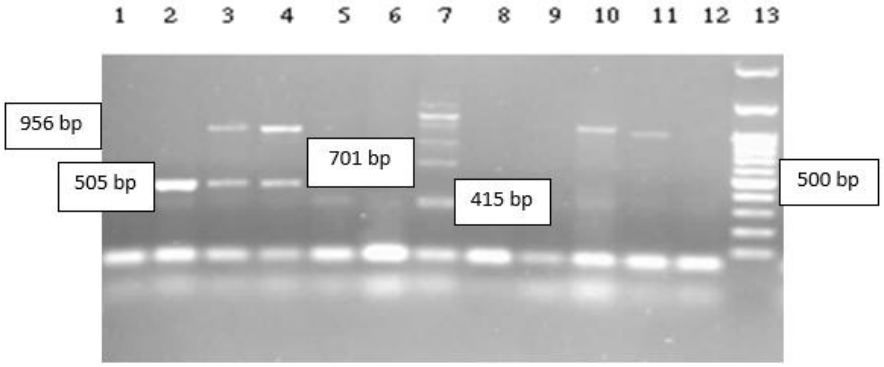

Figure 3. Multiplex electrophoresis PCR of tetracycline (*tet*) genes, product sizes are *tetB* = 415bp, *tetC* = 505 bp, *tet39* = 701bp, *tetA* = 956 bp. Well 13: Molecular marker (100bp), band size from bottom to top -100 bp - 200 bp - 300 bp -400 bp -500 bp (sharp band) - 600 bp - 700 bp - 800 bp - 900 bp -1000bp

4.Discussion

Klebsiella pneumoniae is a gram-negative intestinal bacterium that forms part of the natural flora of the human body. This bacterium causes a wide range of diseases, including bacteremia, pneumonia, UTI, liver abscesses, and sepsis. In recent decades, due to the indiscriminate and unscientific use of antibiotics, we have witnessed the emergence and spread of drug-resistant strains in this bacterium. In 2014, the World Health Organization (WHO) reported *K. pneumoniae* as one of three antibiotic-resistant pathogens. The global spread of drug-resistant strains is a serious threat to global health **(23, 27)**.

Among the antibiotics studied in this study, erythromycin and tetracycline demonstrated the highest resistance, with ceftazidime and cefotaxime the lowest resistance. According to Heidari *et al.* (2018), the resistance of *K. pneumoniae* to ceftazidime ratio was 55.7%. Even though this antibiotic is a powerful tool against *K. pneumoniae*, the resistance to ceftazidime is high. This difference can be due to the indiscriminate use of antibiotics in different geographical areas **(28)**.

Klebsiella pneumoniae is the most common species of *Klebsiella*, which causes human infections. By producing beta-lactamase, it causes hydrolysis and inactivation of most beta-lactam antibiotics such as penicillins, cephalosporins and monobactams. But it does not affect cephamycin and carbapenem. Carbapenems were among the antibiotics used to treat drug-resistant infections in the past. But today, they are a health threat due to the production of carbapenemases by *Enterobacteriaceae* **(26)**.

Our results showed that out of 100 isolates of *K. pneumoniae*, 12 samples were resistant to cefotaxime and 15 to ceftazidime. While a 2016 study by Ribeiro *et al.* on 75 samples of *Klebsiella* showed that all isolates were resistant to ceftazidime and cefotaxime **(29)**. In the present study, cefotaxime and ceftazidime were reported to be effective antibiotics. The results of these two studies were inconsistent, which could be due to the indiscriminate use of these antibiotics and the emergence of new resistant strains or the acquisition and transfer of plasmid resistance genes . Multiplex PCR methods for detecting *tet* resistance genes have not been investigated so far, and this is the first research in this regard. In 2017, Taitt *et al.* performed phenotypic and genotypic antibiotic resistance tests on 87 *K. pneumoniae* specimens. The results showed that more than 2.3 of the isolates are resistant to 5 or more antibiotics. This is a threat to public health and must be considered a critical challenge by the Centers for Disease Control and Prevention **(30)**.

Most isolates were resistant to tetracycline, which was consistent with the recent study that reported 44% tetracycline resistance, which may be due to the proximity of these isolates in these two regions. The results obtained from the frequency of tetracycline genes in the present study are slightly consistent with the study of this group. The study reported the frequency of *tetA* and *tetB* genes to be 16% and 9%, respectively **(31)**. While in the present study, the frequency of *tetA* genes is 39%, and *tetB* is 8%. The prevalence of the *tetA* gene has been reported to be higher, which may be due to mutations in the gene, but the prevalence of the *tetB* gene is almost equal and consistent.

The study by Bokaeian *et al.* (2014) was conducted on 30 samples of *K. pneumoniae*. The results of this group showed that the isolates were resistant to erythromycin (70%), cefixime (53%), tetracycline (50%), and ceftazidime (36%). All samples were positive for *tetA* and *tetB* genes. In the present study, all samples were resistant to erythromycin (93%), tetracycline (42%), cefotaxime (12%), and ceftazidime

(15%). Moreover, 39 samples tested positive for the *tetA* gene and 8 isolates for the *tetB* gene. In both studies, erythromycin was reported to be the most resistant antibiotic in this respect. But in other cases, more than our results, it was reported that this discrepancy is due to incorrect administration of antibiotics in the treatment of infections or transfer of resistance genes by various transport agents such as integrons, plasmid R, transposons, and bacteriophages **(32)**.

Another study was conducted by Kashefieh *et al.* (2019) on 100 samples of *K. pneumoniae*. The frequency of 42%, 30%, 16%, and 21% were reported for *tetB*, *tetA*, *tetC*, *tetD*, respectively. The prevalence of the *tetA* gene is consistent with the present study, but the frequency of other genes has been reported to be more. This discrepancy may be due to the indiscriminate use of antibiotics in different geographical areas **(33)**.

Antibiotic resistance genes are found in gram-negative and gram-positive bacteria. In the Adelowo study, which reported 13 microorganisms from contaminated water, *tetA*, *B*, and *C* were not observed, and 8 isolates (three gram-positive and five gram-negative bacteria) were positive for *tet39*. In a recent study, 8 isolates tested positive for the *tet39* gene. Therefore, the two studies are consistent.

The absence of other genes in the isolates may be due to limited samples. Tetracycline resistance is the most common type of antibiotic resistance that requires monitoring of *tet39* resistance gene diversity **(34)**. Adding antibiotics (tetracycline, erythromycin, streptomycin, and beta-lactamases) to animal feed is considered a source of antibiotic resistance that poses challenges in treating human and animal infections **(35)**.

5. Conclusion

The results of this study reported resistance of *K. pneumoniae* isolates to erythromycin and tetracycline antibiotics. Most samples were positive for 2 tetracycline resistance genes, and none of the 4 resistance genes were observed simultaneously.

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Conflict of Interest

The authors declared no conflict of interest.

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