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ORIGINAL ARTICLE

Molecular Evaluation of β-Lactamase (*bla$_{SHV}$*, *bla$_{TEM}$*, *bla$_{CTX-M}$*) Genes in Multidrug Resistant of *Klebsiella Pneumoniae*

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SUMMARY

Background: ***Klebsiella*** **is an opportunistic pathogen, which is the most common causes of nosocomial infections. To date, the prevalence of ESBL-producing pathogens has increased and is associated with mortality, morbidity, and healthcare costs. The aim of this investigation was to determine the frequency of *bla$_{SHV}$*, *bla$_{TEM}$*, and *bla$_{CTX-M}$* genes from *Klebsiella pneumonia* isolated from patients with UTI in the city of Qom.**
Methods: **In the cross-sectional study, a total of 500 urinary samples were cultured in MacConkey agar and identified using the biochemical test. For a total of 340 positive *K. pneumonia* samples the antimicrobial susceptibility was determined using the Kirby-Bauer disc diffusion approach. For molecular genotyping, the frequencies of *bla$_{SHV}$*, *bla$_{CTX-M}$*, and *bla$_{TEM}$* genes were determined using a polymerase chain reaction (PCR) method.**
Results: **Our finding revealed that a total of 340 *K. pneumonia* isolates 110 isolates (32.35%) were ESBL producers by the phenotypic method. All of these isolates were assessed by PCR for *bla$_{SHV}$*, *bla$_{CTX-M}$*, and *bla$_{TEM}$* genes. The PCR results demonstrated that the frequencies of *bla$_{TEM}$*, *bla$_{CTX-M}$*, and *bla$_{SHV}$* genes were 59.09% (65 isolates), 74.54% (82 isolates), and 74.54% (82 isolates), respectively.**
Conclusions: **According to our findings, with the higher prevalence of ESBL-producing isolates in the clinical, early detection, and follow-up procedures are critical strategies to the prevention of the spread of multidrug resistant isolates.**
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KEYWORDS

Klebsiella pneumoniae, ESBL, *bla$_{SHV}$*, *bla$_{CTX-M}$*, *bla$_{TEM}$*

LIST OF ABBREVIATIONS

UTI - Urinary tract infection
ESBLs - Spectrum b-lactamases
TEM - Temoneira
SHV - Sulfhydryl variable
CTX-M - Cefotaxime hydrolyzing capabilities
AST - Antibiotic susceptibility testing
LB - Luria-Bertani

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INTRODUCTION

Urinary tract infection (UTI) is one of the most common clinical features of infectious diseases between adults, which affect several million people worldwide [1]. The wide range of uropathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Staphylococcus saprophyticus* are considered the most important bacterial infections, which caused UTI in the community [2]. Clinically, all subjects with UTI are mostly treated with antibiotics, which can be lead to changes of the normal bacterial population of the vagina and gastrointestinal tract and formation of multidrug-resistant bacteria [3]. Due to the high recurrence and multidrug resistance between uropathogens, UTI is an important public health problem in all ages. *K. pneumonia*, from the uropathogens family, is one of the most common infection agents in both nosocomial and community-acquired UTIs [4]. Several spectrum β-lactamase (ESBLs) types have been identified form K. pneumonia isolates, which are related to the plasmids that contain multidrug-resistant determinants. The A type of ESBLs include temoneira (TEM), sulfhydryl variable (SHV), and cefotaxime hydrolyzing capabilities (CTX-M), which are the most common. TEM and SHV types are created from TEM-1, TEM-2, and SHV-1, which are determined by single amino-acid changes [5]. Currently, the β-lactamase genes determined in the hospital isolates were bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ types [6,7]. SHV β-lactamase revealed increased levels of resistance to ceftazidime, whereas CTX-M β-lactamase confers higher levels of resistance to cefotaxime and ceftriaxone than ceftazidime [8]. The frequency of CTX-M subtype variable has been reported in different geographic regions and investigations [9]. In the last years, according to the investigation reports, the epidemiology of ESBLs has significantly increased worldwide [9,10]. Due to the high frequencies of TEM, SHV, and CTX-M type of ESBLs, there is a dangerous signal to the clinical usage of next generation cephalosporins for infectious diseases [11]. As mentioned above, ESBLs as significant transferable multidrug-resistant bacteria such as *K. pneumonia*, is now a critical important cause in public health. To date, there are still discrepancies in investigation reports on the frequencies of ESBL-producing *K. pneumonia* in public health centers of Iran. Furthermore, we aimed to assess the ESBL frequencies of *K. pneumonia* bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ types isolated from subjects suffering with UTI and discriminate the pattern of antimicrobial multidrug-resistance in the hospitals of Qom, Iran.

MATERIALS AND METHODS

Study design and bacterial isolates

Our cross-sectional investigation was conducted on samples from patients with UTI (age range: 10 to 85 years old), which had been referred to the multicenter of hospitals of Qom, Iran, during March 2016 to July 2018. Out of 3,234 urine samples, 1,742 culture plates had isolated bacteria. A total of 340 clinical isolates of *K. pneumonia* which were confirmed with standard biochemical and microbiological tests and included in the assessment. Next, for the identification of the isolates, all samples were conserved in Tryptic Soy Broth (TSB; Merck, Germany) containing 4% glycerol.

Antimicrobial susceptibility testing

For the antibiotic susceptibility testing (AST) to determine imipenem-resistant strains, we used the Kirby-Bauer disk diffusion approach on Mueller-Hinton agar (Merck, Germany) and 10 μg imipenem discs (MAST, England) in accordance with the Clinical and Laboratory Standards Institute (CLSI) guideline. In our study, for quality control, we used *K. pneumoniae* and *E. coli* ATCC 25922 ATCC 700603 as positive and negative controls, respectively.

Double disk synergy test for ESBL identification

To reveal MBL-producing strains resistant to imipenem, we were applied DDST (double disk synergy test) on Mueller Hinton agar medium. In the first step, a 10 μg imipenem disc with an imipenem disk 10 μL EDTA (0.5 mM) placed at 20 mm on Mueller-Hinton agar. Next, the plate was incubated for 18 hours at 37°C. Then the inhibition regions were compared with each other and those with ≥ 7 mm were considered MBL-positive strains [12].

Genomic DNA extraction and molecular analysis of β-lactamase genes

To determine genotype frequencies of *K. pneumoniae* isolates, we cultured bacteria in Luria-Bertani (LB) broth (Hi-Media, Mumbai, India) at 37°C for 24 hours. Next, genomic DNA was extracted directly by boiling method [13].

To assess quantity and quality of the genomic DNA extracted, we used a Nanodrop spectrophotometer (ND-1,000; Thermo Scientific; Wilmington, DE, USA) and agarose gels, which were visualized using a Gel Documentation System (ATP, Iran). In addition, we performed Polymerase Chain Reaction (PCR) to determination of *β-lactamase* gene frequencies (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$) according to a previous report [14]. The sequence specific primers were used for the *β-lactamase* gene amplification are shown in Table 1.

PCR was performed using 150 ng of genomic DNA in a final volume of 25 μL PCR reaction mixture including 12.5 μL Master mix (2 X Red; Amplicon Co., Denmark), 10 pmol of each primer (Faza Biotech, Iran), and 5.5 μL sterile distilled water. Amplification conditions were carried out following the program of the thermal gradient cycler (Eppendorf Co., Germany) shown in Table 2.

In order to separate the PCR amplified product, we used 1% agarose gel electrophoresis at 100 V for 1 hour.

Finally, the PCR products were stained by ethidium

Table 1. The sequence specific primers were used for the *β-lactamase* gene amplifications.

Size of products (bp)	Sequences 5' → 3'	Primer name
SHV-F	ATTTGTCGCTTCTTTACTCGC	1,051
SHV-R	TTTATGGCGTTACCTTTGACC	
TEM-F	CGTTTTCCAATGATGAGCAC	442
TEM-R	CCATCCAGTCTATTAATTGTTGC	
CTX-M-F	AAACTTGCCGAATTAGAGCG	605
CTX-M-R	TTATCCCCCACAACCCAG	

Table 2. PCR programs were used to the *β-lactamase* genes amplification.

β-lactamase genes	Consisting of program	Repeat of cycles
bla_{SHV}		
	2 minutes 95ºC initial denaturation 30 seconds 95ºC denaturation 30 seconds 55ºC annealing 110 seconds 72ºC extension 5 minutes 72ºC final extension	33
bla_{TEM}		
	2 minutes 95ºC initial denaturation 30 seconds 95ºC denaturation 30 seconds 55ºC annealing 50 seconds 72ºC extension 5 minutes 72ºC final extension	33
bla_{CTX-M}		
	2 minutes 94ºC initial denaturation 30 seconds 94ºC denaturation 30 seconds 55.7ºC annealing 70 seconds 72ºC extension 5 minutes 72ºC final extension	33

Table 3. Antibiotic resistance patterns in *K. Pneumonia* isolated from UTI patients.

Antibiotic	Susceptible		Intermediate		Resistant	
	Na	%	Na	%	Na	%
Imipenem	31	9.12	44	12.94	265	77.94
Cefotaxime	137	40.30	35	10.29	168	49.41
Meropenem	48	14.12	30	8.83	262	77.05
Cefepime	110	32.35	23	6.77	207	60.88
Piperacillin	72	21.17	69	20.30	199	58.53
Nitrofurantoin	256	75.30	37	10.88	47	13.82
Tetracycline	173	50.88	37	10.88	130	38.24
Ciprofloxacin	105	30.88	37	10.88	198	58.24
Nalidixic acid	191	56.18	108	31.76	41	12.06
Amikacin	56	16.47	15	4.41	269	79.12
Trimethoprim-sulfamethoxazole	274	80.59	15	4.41	51	15
Ceftazidime	157	46.18	24	7.05	159	46.77

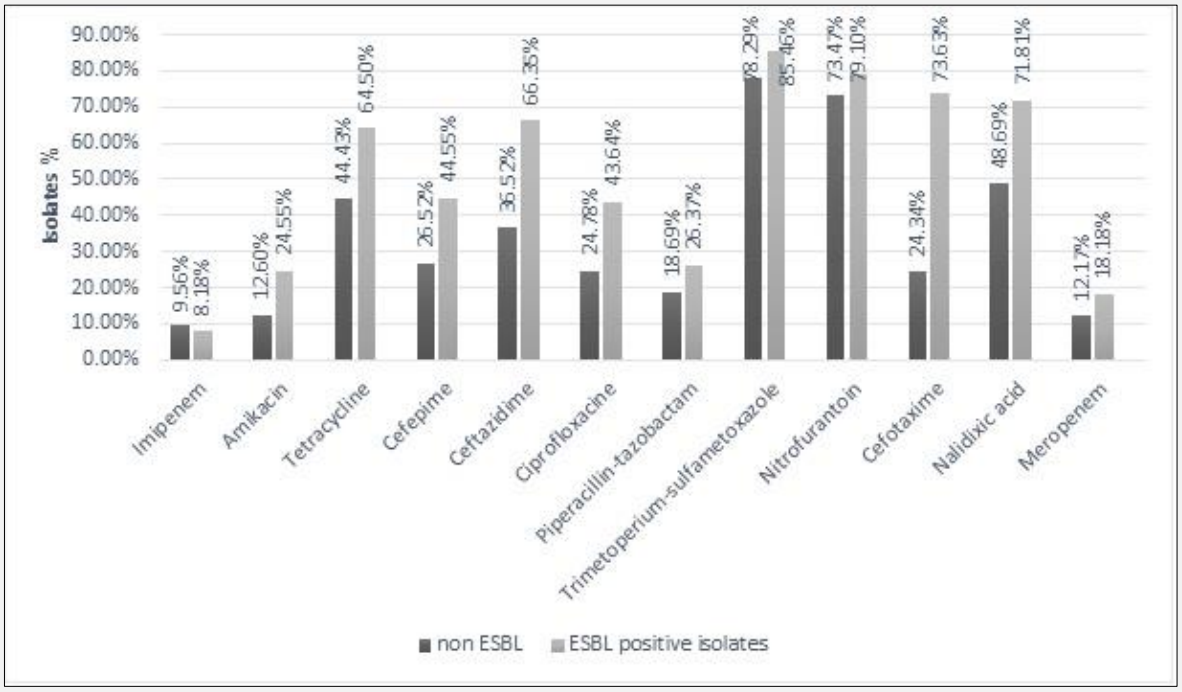

Figure 1. The susceptibility rates of antibiotics to the ESBL positive and negative strains.

bromide (EtBr) and visualized by using ultraviolet light.

Statistical analysis

To analyze our findings, we used SPSS version 20.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA).

RESULTS

In our investigation, of 1,742 urine samples of patients with positive UTI, 340 *K. pneumoniae* isolates were collected and 110 (32.3%) isolates of them were ESBL producers and used for the assessment. The mean age was 47 years old and the range of patients' ages were from 10 months to 85 years old. There were 58% females and 42% males. The prevalence of UTI was high in the age group 18 - 50 years (64%). Low frequencies were seen in the < 2-year-old (3%) group.

In our investigation, the ESBL positive isolates were assessed using DDST and disk diffusion methods. The findings of disk diffusion susceptibility testing of patients with UTI showed that the *K. pneumoniae* isolates were predominantly resistant (80.59%) to Trimethoprim-sulfamethoxazole and showed low susceptibility rates (9.12%) to Imipenem. The susceptibility of isolates to other antibiotics was: cefotaxime 40.30%; Meropenem 14.12%; Cefepiem 32.35%; Piperacillin/tazobactam 21.17%; Nitrofurantoin 75.30%; Tetracycline 50.88%; ciprofloxacin 30.88%; nalidixic acid 56.18%; Amikacin 16.47%, Ceftazidime 46.18%. The findings also declared that the ESBL-producing strains showed different sensitivities to 12 antibiotics in Table 3. In addition, the susceptibility rates of antibiotics to the ESBL positive and negative strains were shown in Figure 1. To molecular detection of ESBL-producing isolates (n = 110), which were assessed using the combination disc test were included for the determining of $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} encoding gene frequencies by PCR. Molecular distribution analyses of the ESBL-producing isolates showed that bla_{SHV} gene is the most prevalent ESBL type (74.54%), followed by $bla_{CTX\text{-}M}$ (74.54%), and finally bla_{TEM} (59.09%) (Figures 2 - 4). In addition, our findings showed that 40 isolates (37.27%) carried the three $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} genes, 41 (39.10%) isolates contained both $bla_{CTX\text{-}M}$ and bla_{SHV} genes, and 20 (18.18%) possessed the single $bla_{CTX\text{-}M}$ gene. Importantly, 6 isolates (5.45%) did not have any of the 3 genes assessed.

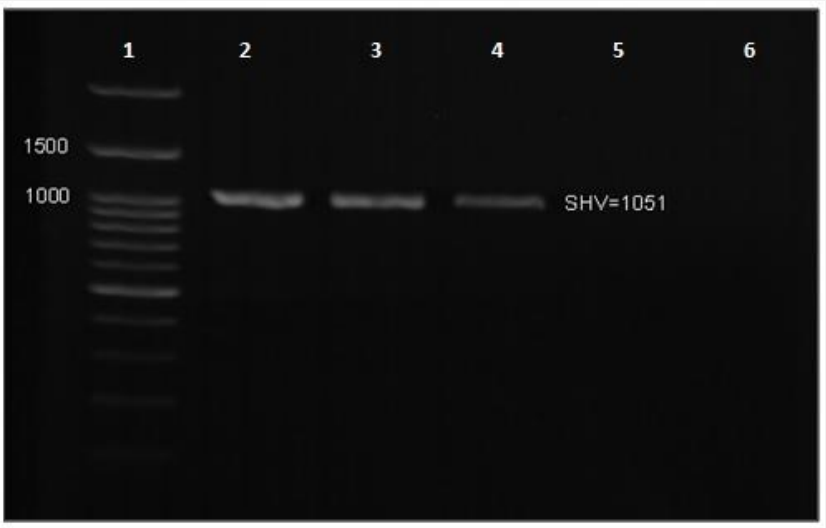

Figure 2. Analysis of gene encoding *bla*$_{SHV}$ in ESBL-producing *K. pneumoniae.*

Lane 1 - DNA size marker (100 bp), Lane 2 - Positive control (K. pneumoniae ATCC 700603), Lanes 3 and 4 - The 1,051 bp PCR product of *bla*$_{SHV}$, Lane 5 - Negative control (E. coli ATCC 25922), Lane 6 - Non template control.

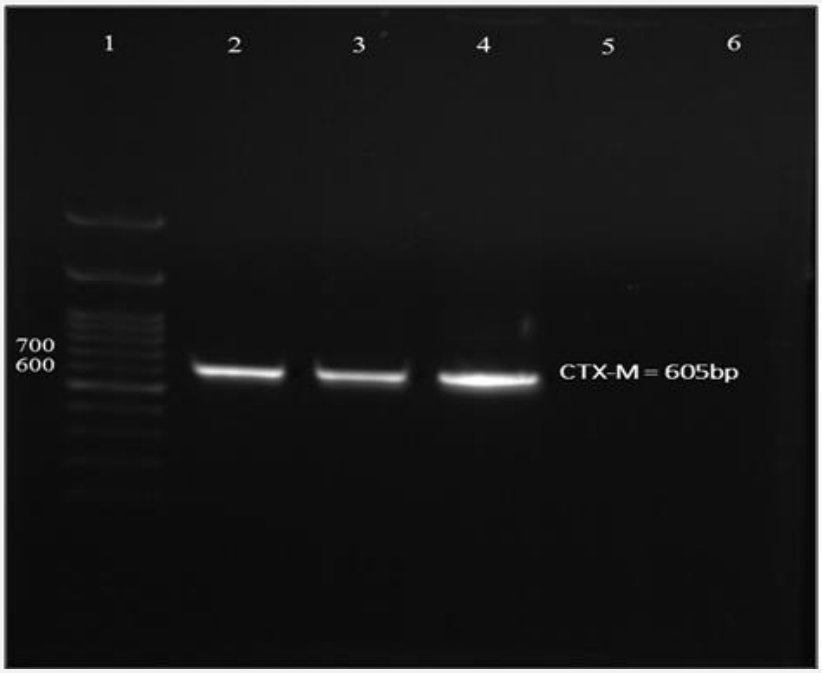

Figure 3. Analysis of gene encoding *bla*$_{CTX-M}$ in ESBL-producing *K. pneumonia.*

Lane 1 - DNA size marker (100 bp), Lane 2 - Positive control (K. pneumoniae ATCC 700603), Lanes 3 and 4 - The 605 bp PCR product of *bla*$_{SHV}$, Lane 5 - Negative control (E. coli ATCC 25922), Lane 6 - Non template control.

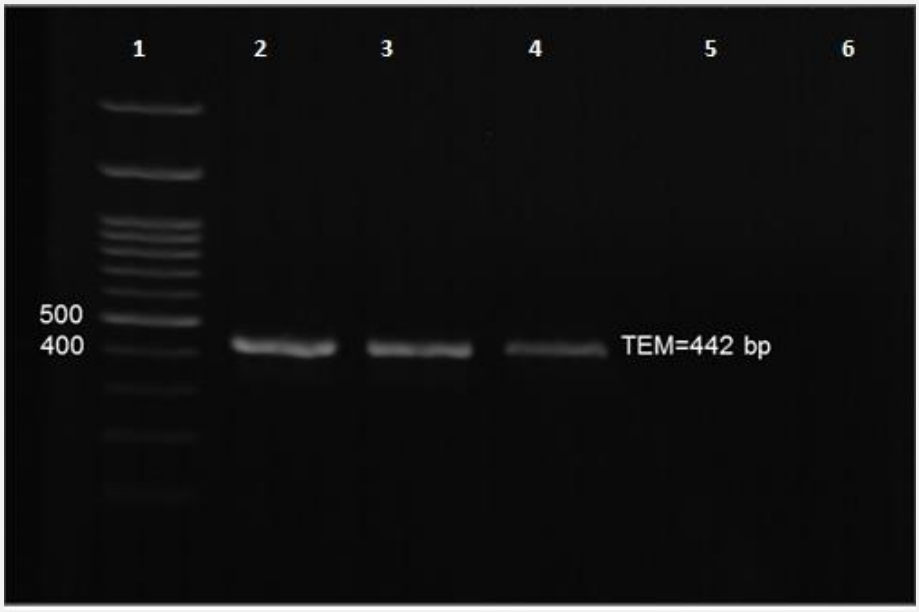

Figure 4. Analysis of gene encoding *bla*$_{TEM}$ in ESBL-producing *K. pneumonia.*

Lane 1 - DNA size marker (100 bp), Lane 2 - Positive control (K. pneumoniae ATCC 700603), Lanes 3 and 4 - The 442 bp PCR product of *bla*$_{SHV}$, Lane 5 - Negative control (E. coli ATCC 25922), Lane 6 - Non template control.

DISCUSSION

Klebsiella pneumonia is one type of gram-negative bacteria which causes infections by producing β-lactamase enzymes and is related to high morbidity and mortality rates worldwide [15]. The aim of the present investigation was to assess the frequencies of antibiotic resistance genes (β-lactamase including: *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ genes) and antibiotic susceptibility rates by molecular method such as PCR. Knothe et al first reported on *K. pneumoniae* isolates with multi-drug resistance [16]. Previous investigations have reported on the high prevalence of β-lactamase-producing *K. pneumoniae* in several countries [17-21]. Of note, antibiotic resistance is carried on transmissible elements, which can be transferred to other bacteria. In the current investigation, we determined the susceptibility rates of 340 UTI specimens of clinical *K. pneumonia* strains against twelve antibiotics. The findings revealed that high resistance was seen for Trimethoprim-sulfamethoxazole (80.59%), Nitrofurantoin (75.30%), Nalidixic acid (56.18%), Tetracycline (50.88%), and Ceftazidime (46.18%). Previous investigations reported rates that were different to the rates revealed in this study. In addition, our findings revealed that high susceptibility (84.55%) to imipenem is in agreement with those reported in previous investigations [15,22-25]. Multi-drug resistant (MDR) refers to the strains that are resistant to more than two classes of antibiotics [26]. Surprisingly, in our findings, 88% of isolates were included in the MDR group. This report contrasts with those published by other investigations [15,23]. Of a total of 300 MDR isolates, 104 isolates were reported as *β-lactamase*-positive (35%). These findings are similar to other published studies [27,28]. However, the frequencies of *β-lactamase*-producing *Klebsiella spp* are reported between in the range of 20% - 79% in different geological regions. In our investigation, we aimed to declare the frequencies of *β-lactamase* genes in clinical *K. pneumoniae* isolates. The higher frequency was detected in 74.54% (82/110) and 74.54% (82/110) for *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes respectively. In addition, *bla*$_{TEM}$ was reported at 59.09% (65/110) of the isolates.

Sedighi et al. reported the frequencies of *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX}$ genes at 37.8%, 24.3%, and 18.9%, respectively [15]. Monstein et al. determined the frequency of *bla*$_{SHV}$ gene in 8.1%, *bla*$_{SHV}$ in 18.9%, and *bla*$_{TEM}$ in 2.7% of the isolates, and the combination of all three (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$) in 8% of the isolates [29]. These findings are in disagreement with those reported in the present study. In addition, Hassan and Abdalhamid reported different frequencies of *bla*$_{CTX-M}$ (97.4%) in comparison to *bla*$_{SHV}$ (23.1%) gene [30]. Furthermore, in previous investigations, as well as in our inves-

tigation, the three bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes showed higher frequencies of β-lactamase genes in *K. pneumoniae* isolates. Our findings suggest significant high frequencies of bla_{CTX-M}, bla_{SHV} and bla_{TEM} genes in *K. pneumoniae* isolates gathered from multicenter of hospitals of Qom, Iran.

According to our investigation, the findings demonstrate that the MDR isolates which carry the most common resistance genes, is significantly worrying and a platform needs to be created for antibiotic susceptibility surveillance to evaluate infections caused by *K. pneumoniae* in Iran. Due to the high frequencies of β-lactamase, this may play a critical role in the increasing antimicrobial susceptibility rate and decreased mortality rate caused by resistant bacterial infections using β-lactamase genes screening. The current investigation has limitations in the evaluation of MDR bacteria. One of the most important issues is related to the other β-lactamases family genes, which can lead to increasing antibiotic resistance rates. In addition, due to the financial constraints, we could not to reveal the molecular mechanisms of the gene target.

In conclusion, it is possible to design a control infection platform decreasing of spread of MDR bacteria. In addition, using combination therapy could be a useful approach to control resistant infections. However, more studies are critical to obtain reliable and effective results related to antibiotic susceptibility surveillance.

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Declaration of Interest:
The authors declare that they have no conflicts of interest.

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