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Research Article

Frequency of beta-lactamase genes (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$) in multidrug-resistant *Klebsiella pneumoniae*

Masoumeh Lak Hondori[1], Maryam Mohammadi-Sichani [1,*], Mozhgan Ghiasian [1]

1. Department of Microbiology, Fal. C., Islamic Azad University, Isfahan, Iran

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ABSTRACT

The global spread of multidrug-resistant (MDR) strains of *Klebsiella pneumoniae* is a significant public health concern. This study aims to investigate the prevalence of beta-lactamase genes *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *bla*$_{KPC}$, and *bla*$_{OXA-48}$ in MDR *K. pneumoniae* isolates obtained from nosocomial infections in Isfahan, Iran. Ninety-six *K. pneumoniae* isolates were collected from clinical infections. After confirming the identity of the isolates through biochemical tests, their antibiotic sensitivity was assessed using the disk diffusion method. Extended-spectrum beta-lactamase production was evaluated with the combination disk method, utilizing cefotaxime and cefotaxime/clavulanic acid. The presence of beta-lactamase genes was detected using specific primers and PCR. All isolates were resistant to amoxicillin. Additionally, resistance was observed in 56.25% of isolates to imipenem, 57.29% to cephalothin, 52.08% to ciprofloxacin, and 52.08% to cefotaxime. Conversely, the highest sensitivity was recorded for tetracycline (64.58%), followed by chloramphenicol (61.45%), gentamicin (56.25%), and nitrofurantoin (54.16%). Phenotypic testing revealed that all 96 isolates were positive for broad-spectrum beta-lactamases. Moreover, 44 isolates were classified as MDR, exhibiting resistance to at least three different antibiotic classes. The frequencies of the *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes among MDR strains were 45.45%, 50%, 72.72%, 84.09%, and 86.36%, respectively. The presence of MDR isolates and broad-spectrum beta-lactamase genes, particularly in *K. pneumoniae*, highlights the need for cautious management of these infections.

1. Introduction

Klebsiella pneumoniae is a Gram-negative bacterium known for causing various infections and increasingly resisting antibiotics. Its resistance is largely due to the production of Extended-Spectrum Beta-Lactamases (ESBLs), which break down a wide range of beta-lactam antibiotics, including penicillins and cephalosporins, making them ineffective. The genes for ESBL production are typically found on plasmids, allowing for rapid spread of resistance among bacteria (Kadivarian et al., 2023; Shrestha et al., 2022). ESBL-producing strains of *K. pneumoniae* cause various infections, including UTIs, respiratory, and bloodstream infections. Their presence complicates treatment and contributes to nosocomial infections in healthcare settings, making patient care more challenging (Raouf et al., 2022).

*Corresponding author: Maryam Mohammadi-Sichani
E-mail address: ma.mohammadi1347@iau.ac.ir

ESBLs in *K. pneumoniae* not only break down penicillins and early cephalosporins but also more advanced cephalosporins and monobactams. The production of both ESBLs and carbapenemases by some strains greatly limits treatment options, making infections harder to manage and posing a significant global health challenge (Bastidas-Caldes et al., 2023; Chu et al., 2024). Combating ESBL-producing *K. pneumoniae* requires a multi-faceted approach, including surveillance, strict infection control, and careful antibiotic use. Ongoing research into new treatments and antibiotics is crucial. Understanding the genetic mechanisms and spread of ESBLs is essential for managing infections and preserving current antibiotics (Carvalho et al. 2021; Davood et al. 2016). Therefore, this study was conducted to determine the frequency of major beta-lactamase genes (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *bla*$_{KPC}$, and *bla*$_{OXA-48}$) among multidrug-resistant *K. pneumoniae* isolates from nosocomial infections in Isfahan, Iran.

2. Materials and Methods

2.1. Collection and identification of samples

In this descriptive study, a total of 96 clinical isolates of *K. pneumoniae* were collected from various clinical specimens, including urine, tracheal aspirates, blood, and others, over a six-month period (January–July 2023) in Isfahan, Iran. All isolates were cultured on Blood agar. After observing mucoid colonies and performing Gram staining, a series of biochemical tests were conducted. These tests included oxidase, catalase, motility, citrate utilization, Triple Sugar Iron (TSI), urease, Methyl Red (MR), and Voges-Proskauer (VP) tests (Osman et al., 2020). The final identification of *K. pneumoniae* isolates was confirmed using polymerase chain reaction (PCR) with universal primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3')(Wu et al., 2021).

2.2. Determination of antibiotic sensitivity

The sensitivity of *K. pneumoniae* isolates was assessed using the Kirby-Bauer method, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2023). The antibiotic disks used included ciprofloxacin (5 µg), cefotaxime (30 µg), cephalothin (30 µg), gentamicin (10 µg), tetracycline (30 µg), cefixime (9 µg), imipenem (10 µg), chloramphenicol (30 µg), nitrofurantoin (200 µg), and amoxicillin (25 µg) (Padtan Teb Co., Iran). The isolates were categorized as sensitive, intermediate, or resistant based on the diameter of the inhibition zones. Each sensitivity test used *K. pneumoniae* ATCC 700603 as a quality control. An isolate was classified as multidrug-resistant (MDR) if it exhibited resistance to at least three classs of antibiotics (Wayne 2013).

2.3. Phenotypic Detection of ESBL

The production of ESBLs in *K. pneumoniae* isolates was confirmed using the Disk Combination method. This involved the use of discs containing cefotaxime (30 µg) and cefotaxime/clavulanic acid (30/10 µg). The tests were interpreted according to CLSI guidelines. A difference of ≥5 mm in the inhibition zone between cefotaxime alone and in combination with clavulanic acid indicated the presence of ESBL production. *E. coli* ATCC 25922T and *K. pneumoniae* ATCC 700603 were used as ESBL-negative and positive strains, respectively (Rawat et al. 2010).

2.4.Molecular identification of ESBL

The boiling method was used for bacterial DNA extraction. Both quantitative and qualitative assessments were conducted after the extraction process. For quantitative analysis, the concentration of DNA samples was determined using a nanodrop, measuring optical absorption at 216 and 296 nanometers. The ratio of absorbance at 216 nm to 296 nm was used to assess DNA concentration. For qualitative assessment, the extracted DNA samples were analyzed using gel electrophoresis, and the percentage of agarose gel was observed. The supernatant containing the extracted DNA was carefully transferred to a clean tube for downstream applications (Alshahrani et al., 2022). To amplify the desired fragment and investigate the presence of the *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M-15}$, *bla*$_{KPC}$, and *bla*$_{OXA-48}$ genes, specific primers were used as outlined in Table 1 (Kao et al. 2016). The primers (Table 1) were synthesized by Roya-Biogene Company (Tehran, Iran).

Table1. Characteristics of beta-lactamase primer sequences.

Target gene	name	Sequences	Amplicon (bp)	Accession No.
SHV	FSHV1 RSHV1	5'-TGGGAAACGGAACTGAATGAG-3' 5'-TCGTCCACCATCCACTGCAG-3'	147	EF035566.1 OP253964.1
TEM	FTEM1 RTEM1	5'-CACCAGTCACAGAAAAGCATC-3' 5'-GTTAGCTCCTTCGGTCCTCC-3'	130	AY392531.1 MG653126.1
OXA-48	FOX48 ROX48	5'-ACAAGCCATGCTGACCGAAG-3' 5'-CCACACATTATCATCAAGTTC-3'	124	OQ133354.1 OQ133347.1
KPC	FKPC RKPC	5'- TCTTGCTGCCGCTGTGCTGG-3 5'- CCACCGTCATGCCTGTTGTC-3'	134	MZ570431.1 OQ579138.1
CTX-M	FCTXM15 RCTXM15	5'-ATGGGACGATGTCACTGGCTG-3' 5'-GAACGTTTCGTCTCCCAGC-3'	140	KC699839.1 KC699838.1

Amplification was carried out using a thermocycler (Eppendorf, Germany) in a 25 µL reaction mixture containing 2.5 µL of 10X PCR buffer, 0.75 µL of 50 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 0.5 µL of each primer (10 pmol/µL), 1 µL of template DNA, 0.25 µL of Taq DNA polymerase (5 U/µL), and 18 µL of nuclease-free water. The reaction mixtures were initially denatured at 94 °C for 5 minutes, followed by 35 cycles consisting of 30 seconds at 94 °C, 40 seconds at 55 °C, and 30 seconds at 72 °C. A final extension step at 72 °C for 5 minutes was then performed. PCR products were visualized by 1.5% agarose gel electrophoresis at 85V and 390mA, followed by staining with DNA Green viewer dye (Estabraghi et al. 2016). The PCR products were sent to Roya-Biogene Company for DNA sequencing. The obtained DNA sequences were analyzed using Chromas (version 2.1.1) and aligned against the NCBI database using the nucleotide BLAST server (Aslam et al. 2022; Saki et al. 2022).

3. Results

The antibiotic resistance of *K. pneumoniae* isolates was determined using the disk diffusion method. The results of the antibiogram for the various antibiotics are presented in Fig1.

The highest level of resistance was observed against amoxicillin, with 100% of the isolates showing resistance. Additionally, 56.5% of the isolates were resistant to imipenem, 57.3% to cephalothin, and 52.1% to both ciprofloxacin and cefotaxime. Moreover, resistance was observed in 39.6% of the isolates to nitrofurantoin, 38.5% to gentamicin, 36.5% to chloramphenicol, and 33.3% to tetracycline. The antibiogram results indicate that more than 50% of the isolates were resistant to cephalothin, imipenem, ciprofloxacin, and cefotaxime. The analysis of antibiotic resistance revealed that 44 isolates were resistant to at least three different antibiotic classes, categorizing them as MDR isolates. The antibiogram results for these MDR *K. pneumoniae* isolates showed that over 80% were resistant to cephalothin, imipenem, ciprofloxacin, and cefotaxime, a rate significantly higher than that observed in the general set of isolates (Table 2).

Statistical analysis revealed no significant difference in resistance to amoxicillin between the MDR and non-MDR isolate groups. However, significant resistance was observed in both groups for antibiotics such as imipenem, cefotaxime, cephalothin, ciprofloxacin, nitrofurantoin, gentamicin, chloramphenicol, and tetracycline ($p < 0.05$). All *K. pneumoniae* isolates with MDR demonstrated the ability to produce ESBLs based on the phenotypic combination disk test. ESBL production was confirmed by an increase of 5 millimeters or more in the zone diameter around the cefotaxime/clavulanic acid disk compared to the zone diameter around the cefotaxime disk.

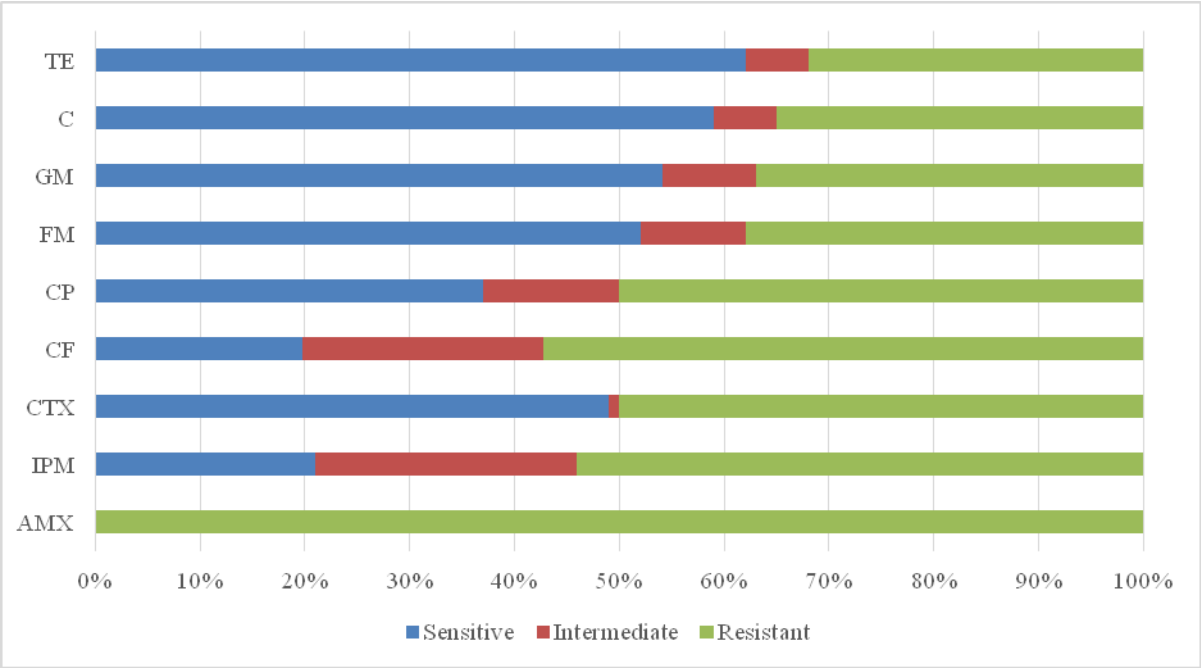

Figure 1. Frequency of antibiotic resistance among *K. pneumoniae* isolates

Table 2. Sensitivity to antibiotics in MDR *K. pneumoniae* isolates and non-MDR isolates.

	Non-MDR *K. pneumoniae* isolates (%)			MDR *K. pneumoniae* isolates (%)		
Antibiotic	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Amoxicillin	52 (100)	0 (0)	0 (0)	44 (100)	0 (0)	0 (0)
Imipenem	19 (36.5)	20 (38.5)	13 (25.0)	35 (79.5)	5 (11.4)	4 (9.1)
Cefotaxime	11 (21.2)	0 (0)	41 (78.8)	39 (88.6)	1 (2.3)	4 (9.1)
Cephalothin	13 (25.5)	18 (34.6)	15 (28.4)	42 (95.5)	4 (9.1)	4 (9.1)
Ciprofloxacin	11 (21.2)	10 (19.2)	31 (59.6)	39 (88.6)	3 (6.8)	2 (4.5)
Nitrofurantoin	4 (7.7)	4 (7.7)	44 (84.6)	32 (72.7)	4 (9.1)	8 (18.2)
Gentamycin	2 (3.8)	7 (13.5)	43 (82.7)	35 (79.5)	2 (4.5)	7 (15.9)
Chloramphenicol	3 (5.8)	1 (1.9)	48 (92.3)	30 (68.2)	5 (11.4)	9 (20.5)
Tetracycline	7 (13.5)	1 (1.9)	44 (84.6)	25 (56.8)	5 (11.4)	14 (31.8)

To identify bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$, bla_{KPC}, and bla_{OXA} genes in clinical isolates of *K. pneumoniae*, PCR was performed using specific primers. The gel electrophoresis results are depicted in Figure 2.

The results of selected beta-lactamase genes in 44 MDR *K. pneumoniae* isolates are presented in Table 3.

The frequency of the bla_{KPC}, $bla_{OXA\text{-}48}$, $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM}, and bla_{SHV} genes in MDR *K. pneumoniae* isolates was 45.5% (20 strains),

50% (22 strains), 72.7% (32 strains), 84.1% (37 strains), and 86.4% (38 strains), respectively. Among these, 8 isolates harbored all 5 beta-lactamase genes, and 9 isolates possessed 4 genes, specifically bla_{TEM}, bla_{SHV}, bla_{KPC}, and $bla_{CTX\text{-}M\text{-}15}$ (Table 3).

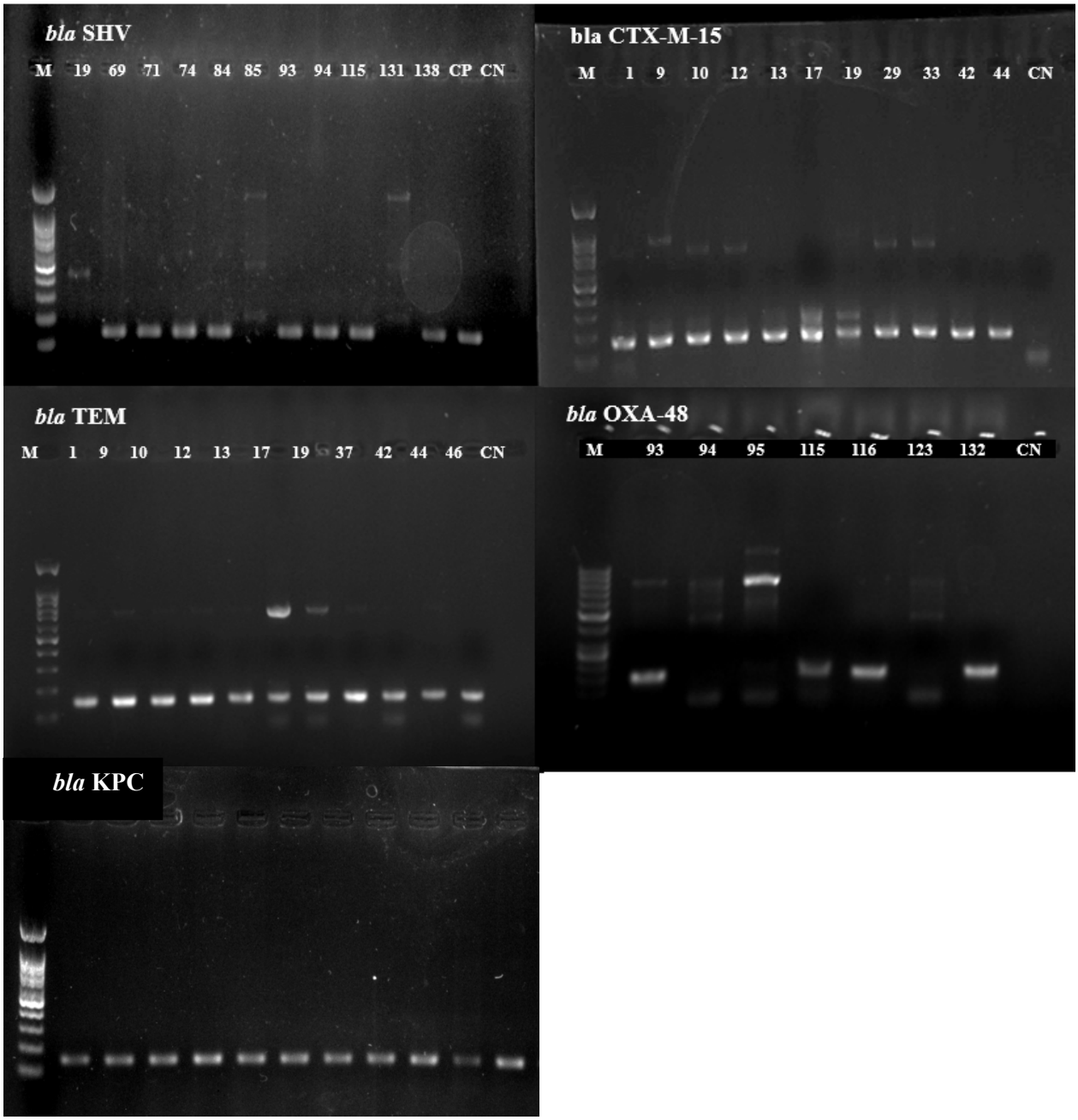

Figure 2. Gel electrophoresis of PCR products for the beta-lactamase resistance gene in some of the isolates. (M: Marker, CP: Positive control, CN: Negative control, and numerical identifiers for the isolates).

Table 3. beta-lactamase genes in 44 MDR *K. pneumoniae* isolates

Isolate No.	ESBL genes				
	bla_{OXA-48}	bla_{SHV}	bla_{TEM}	$bla_{CTX-M-15}$	bla_{KPC}
12, 13, 17, 49, 74, 115, 116, 161	+	+	+	+	+
1, 9, 10, 46, 60, 94, 102, 123, 138, 154	-	+	+	+	-
42, 86, 93, 105	+	+	+	+	-
109, 110, 112, 132, 144	+	+	+	-	+
29, 33	+	+	-	+	-
69, 71	-	+	+	-	-
44, 84, 145	-	+	+	+	+
155, 157	-	+	-	-	-
5	+	+	-	-	-
19	-	-	+	+	-
37	-	-	+	-	-
79	-	-	+	-	+
85	-	-	-	+	-
95	-	-	+	+	+
131	+	-	+	+	+
140	+	+	-	+	+
Total (%)	22 (50.0)	38 (86.4)	37 (84.1)	32 (72.7)	20 (45.5)

4. Discussion

K. pneumoniae has become increasingly resistant to antibiotics due to their overuse and improper application. Rapid identification and appropriate treatment of resistant strains are essential. The prevalence of ESBL-producing *K. pneumoniae* varies geographically, with high rates in Asia, the Middle East, and Latin America, often exceeding 50%, while lower but increasing rates are observed in Europe and North America. Antibiotic use in healthcare and agriculture contributes to these regional differences (Lai et al., 2024).

Studies show varying resistance patterns: Moini et al. (2015) reported 10.3% sensitivity to antibiotics and a 46.6% prevalence of drug-resistant strains, consistent with our findings. Both Moini and Al-Sheboul (2023) noted complete resistance to ampicillin but none to imipenem. Ahanjan et al. (2017) found higher resistance rates, with 63% resistance to gentamicin, possibly due to regional differences in sample sources (Al-Sheboul et al. 2023; Moini et al. 2015). Maleki et al. (2018) found that 44.9% of *K. pneumoniae* isolates from urinary tract infections were resistant to various antibiotics, like our finding of 45.83% MDR isolates, indicating a consistent pattern of resistance linked to the bla_{TEM} gene. Kashefieh et al. (2021) and Sarshar et al. (2021) both reported high resistance rates to ampicillin (96% and 94%, respectively), confirming its widespread ineffectiveness against *K. pneumoniae*. While Kashefieh et al. found the highest sensitivity to tigecycline (9%), Sarshar et al. observed 84% susceptibility to gentamicin, suggesting these antibiotics may still be effective treatment options, though their efficacy varies by region (Kashefieh et al., 2021; Sarshar et al., 2021). Abbasi et al. (2023) and Nakamura-Silva et al. (2022) highlighted the increasing challenge of antibiotic resistance. Abbasi et al. found high resistance rates in central provinces to ampicillin (93.8%) and cotrimoxazole (64.4%), with the lowest resistance to tigecycline (1.6%). Nakamura-Silva et al. reported that 61.9% of isolates were MDR and 23.8% were extremely drug-resistant (XDR), with complete resistance to amoxicillin (100%) and a high resistance rate to imipenem (56.25%) (Abbasi et al., 2023; Nakamura-Silva et al., 2022). These studies collectively emphasize the critical and growing issue of antibiotic resistance in *K. pneumoniae*, particularly to commonly used antibiotics like ampicillin, cefotaxime, and ceftriaxone. The varying resistance patterns observed across different regions highlight the need for localized antibiotic stewardship programs and the development of new therapeutic strategies. The continued efficacy of certain antibiotics, such as

imipenem and tigecycline, offers some hope, but the increasing resistance to even these last-line treatments underscores the urgency of addressing this public health challenge.

The distribution of different ESBL types and their antibiotic resistance characteristics can vary by country and even among clinics within the same location. In this study, the frequencies of bla_{KPC}, bla_{OXA-48}, $bla_{CTX-M-15}$, bla_{TEM}, and bla_{SHV} genes in multidrug-resistant strains were 45.45% (20 strains), 50% (22 strains), 72.72% (32 strains), 84.09% (37 strains), and 86.36% (38 strains), respectively. Research in Iran and other regions has explored *K. pneumoniae* strains producing ESBL. For example, the frequency of *K. pneumoniae* beta-lactamase producing isolates was reported as 79.1% in one study and 72% in another which is higher than our findings (Feizabadi et al., 2010).

Roshdi et al. found that 59.2% of *K. pneumoniae* isolates from Azerbaijan were ESBL-positive, with bla_{TEM} and bla_{SHV} genes present in 49.3% and 43.7% of isolates, respectively, and both genes in 7% of samples. Our study's ESBL frequency (45.83%) is similar, but the prevalence of bla_{TEM} and bla_{SHV} genes was nearly double (Roshdi Maleki et al. 2021). Malekjamshidi et al. studied 250 *Klebsiella* isolates in Yazd province, identifying bla_{SHV} as the most common β-lactamase gene, followed by bla_{TEM} and bla_{CTX-M}, while bla_{OXA-48}, bla_{KPC}, and bla_{NDM} were not detected. Ampicillin showed the highest resistance, while imipenem was the most effective (Malekjamshidi et al., 2020). Genetic analysis in our study revealed higher prevalence rates for $bla_{CTX-M-15}$, bla_{TEM}, and bla_{SHV} genes compared to previous studies, indicating an increase in β-lactamase gene prevalence.

Ahanjan found bla_{TEM} and bla_{CTX} genes in 55% and 45% of *K. pneumoniae* isolates, respectively, lower than our findings, possibly due to the increasing abundance of β-lactamase genes over time (Ahanjan et al., 2017). Maleki et al. (2018) reported 25% of *K. pneumoniae* isolates from urinary tract infections were ESBL-positive, while our study shows a higher frequency, likely due to changes in antibiotic use and detection methods (Maleki et al., 2018). Farhadi reported high ampicillin resistance (93%) and high frequencies of the genes bla_{SHV} (91.4%), bla_{TEM} (82.2%), bla_{CTX-M} (79.3%), bla_{KPC} (29.3%), and bla_{OXA-48} (36.2%), consistent with our findings (Farhadi et al., 2021). Sharafkhah et al. found 40% ESBL production in *K. pneumoniae* isolates, with 55% prevalence of the bla_{TEM} gene, lower than the 84.09% in our study, likely due to differences in sample sources, timing, and locations. (Sharafkhah et al., 2022). Ghanbarinasab's results revealed predominant prevalence of OXA-48-like and NDM carbapenemases among Colistin resistant *K. pneumoniae* clinical isolates (Ghanbarinasab et al., 2023).

Globally, ESBL production rates vary. In Asia, the prevalence of ESBL-producing bacteria is generally higher than in Europe and the Americas. For example, Owusu et al. found that 44% of *K. pneumoniae* isolates from a sample of 181 gram-negative bacteria were resistant to multiple drugs. They reported frequencies of ESBL genes as follows: bla_{CTX-M} (81%), bla_{TEM} (73%), and bla_{SHV} (26%), with carbapenemases bla_{OXA-48} (60%) and bla_{KPC} (40%). These findings are like our results (Owusu et al. 2023). In Germany, Xanthopoulou screened 1,000 bloodstream infection samples over four years and found a 72.72% prevalence of $bla_{CTX-M15}$, aligning with our findings (Xanthopoulou et al., 2022). Additionally, Nakamura-Silva et al. in Manaus, Brazil, reported that 61.9% of 21 *K. pneumoniae* strains were MDR and 23.8% were extremely drug-resistant (Nakamura-Silva et al., 2022). Overall, while there are similarities in ESBL prevalence across different studies, variations are evident due to regional differences and study methodologies.

The rise of ESBL-producing bacteria, driven by factors like antibiotic misuse and prolonged hospital stays, is a major concern due to their resistance to multiple antibiotics, including cephalosporins and aminoglycosides. This resistance is spread through gene transfer on large plasmids in healthcare settings. Regular testing for drug resistance and ESBL production is crucial for guiding effective antibiotic selection and managing drug-resistant bacteria, which can help reduce disease severity and treatment costs.

Conclusion

The study found that *K. pneumoniae* bacteria were fully resistant to amoxicillin, with varying resistance rates to other antibiotics. There was a

notable increase in MDR isolates, resistant to at least three antibiotic classes. The growing resistance is attributed to increased gene transfer and antibiotic overuse, emphasizing the need for strict control measures and regular monitoring. The rise of ESBL-producing bacteria, especially with resistance to third-generation cephalosporins, highlights a significant challenge in treatment, calling for new antibiotics and further research on beta-lactamase genes like *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM-1}}$, and *bla*$_{\text{SHV}}$.

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethics approval

This study was approved by the Ethics Committee of Islamic Azad University, Falavarjan Branch (with the code IR.IAU.FALA.REC.1401.022) and can be viewed on the website of the National Ethics System in Biomedical Research (ethics.research.ac.ir).

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Refereces

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