Assessment of the last-resort antibiotics against Extended Spectrum Beta-Lactamase/carbapenemase and biofilm producer *Klebsiella pneumoniae* isolated from hospitalized patients in intensive care units (ICUs), Iran

Running head: Last-resort antibiotics against Extended Spectrum Beta-Lactamase/carbapenemase *Klebsiella pneumoniae*

Abstract

Pneumonia caused by *Klebsiella pneumoniae* (*K. pneumoniae*) is considered one of the most common causes of hospital-acquired infections. We aimed to investigate the activity of tigecycline, azithromycin, and colistin against *K. pneumoniae* isolated from bronchoalveolar lavage (BAL) samples of suspected cases of ventilator-associated pneumonia (VAP) in COVID-19 patients.

In the current study phenotypic and genotypic screening of ESBLs, AmpC beta-lactamases, and carbapenemase enzymes was investigated. the activity of tigecycline, azithromycin, and colistin against ESBL/carbapenemase producer *K. pneumoniae.* Also, assessment of the ability of biofilm formation was performed. Finally, virulence genes were detected by the PCR method.

By phenotypic detection tests 27 (29.6%) out of 91 *K. pneumoniae* isolates were classified as ESBL/carbapenemase-producing *K. pneumoniae* strains. Also, molecular methods showed, all 27 *K. pneumoniae* isolates harbored at least 1 of the ESBL/carbapenemase-related genes. ESBL-associated genes (19.7% bla_{TEM}, 29.6% bla_{SHV}, and 19.7% $bla_{\text{CTX-M}}$) were detected in 91 *K. pneumoniae* isolates. Carbapenemase-related genes were detected in 17.5% of these isolates ($bla_{\text{OXA-48-like}}$ 15.4%, and bla_{NDM1} 2.1%). All of the 27 selected isolates, exhibited biofilm formation ability. In this study, 92.59%, 92.59%, 81.48%, 88.8%, 40.74%, 11.1 %, 22.22%, 18.5%, 14.81% and 33.33% of the ESBL/carbapenemase producer *K. pneumoniae*

isolates carried *entB*, *mrkD*, *fimH*, *Irp2*, *wcaG*, *mrkA*, *rmpA*, *iutA* and *magA* genes, respectively. But *iucA* gene was not present in any of isolates. Tigecycline and colistin were more effective against these isolates. Multilocus sequence typing (MLST) results for four colistin-resistant isolates showed three different sequence types ST: ST3500, ST273, and 2 cases of ST2558.

The rapid emergence and spread of colistin-resistant and Beta-lactamase producer *K. pneumoniae* has resulted in an alarming situation worldwide. The effective antimicrobial activity of tigecycline against *K. pneumoniae* that produce these enzymes may be efficient in hospitalized patients in ICUs with suspected cases of VAP.

Keywords: *K. pneumoniae*, Carbapenem resistance, Extended-spectrum beta-lactamases, IRAN

1. Introduction

Nosocomial-acquired ESBL and carbapenemase-producing *K. pneumoniae* infections are resulted in high morbidity and mortality because of the limited number of antibiotic treatment options (1). As a result, for the treatment of infections caused by ESBL-producing *K. pneumonia*, carbapenems have been considered suitable options for infection control. Capsular serotypes K1 and K2 in *K. pneumoniae* strains, which are the most frequent isolates from patients worldwide, have been identified as risk factors for liver abscess and complicated endophthalmitis (2).

Carbapenems are considered to be the most reliable last-resort treatment for bacterial infections because they are highly effective against many bacterial species and less vulnerable to most beta-lactam resistance determinants (3). The carbapenems are safer to use than other last-line drugs such as polymyxins. For these reasons, the advent and rapid expanse of Carbapenem resistance in all continents, which are considered the last-resort

antibiotics for the treatment of ESBL-producing *K. pneumonia*, constitutes a universal public-healthcare problem (4). The overuse of carbapenems in hospitals has led to an increase in Carbapenem-resistant *K. pneumoniae*. *K. pneumoniae* carbapenemase (KPC)-producing is becoming distressing. Several mechanisms result in resistance to carbapenems, including the production of carbapenemase of class A (*KPC*, *GES*, and others), class B (mainly *IMP*, *VIM*, or *NDM*), and class D (*OXA-48*) and related enzymes. For the Carbapenem-resistant isolates infections treatment, tigecycline which is one of the glycylcycline derivatives of minocycline can be considered the last-resort (5). *K. pneumoniae* isolates which is classified as extensively-drug resistant (XDR) are quickly emerging due to the dissemination of resistance to aminoglycosides, fluoroquinolones, β-lactams, and carbapenems. Newly, XDR strains have progressed to become PDR by acquiring resistance to tigecycline and polymyxin antibiotics (6). XDR and hypervirulent *Klebsiella pneumoniae* (XDR-hvKp) is a new problem for patients in ICUs, which is one of the superbug bacteria that is recognized as a major cause of hospital-acquired infections. It is essential to raise clinical management of beta-lactamase and biofilm producer *K. pneumoniae* infections, signaled as the next superbug in waiting (7). The prevalence of bacterial co-infection with coronavirus disease has been reported in different rate, but it may be as high as 50% in non-survivors (8). According to the current findings, the top bacteria of secondary bacterial infections, which were detected in COVID-19 patients, were *Mycoplasma pneumonia*, *Pseudomonas aeruginosa*, *K. pneumoniae*, *Acinetobacter baumannii*, and *stenotrophomonas maltophila* (9). These findings support the routine use of antibiotics in the management of the treatment of co-infection associated with COVID-19 hospitalized patients in ICUs, which makes them more exposed to nosocomial infections (9). National Institute for Health and Care Excellence recommended antibacterial treatment for high-risk patients with untreated bacterial infections (8). Biofilm

formation and attachment to surface, and capsular polysaccharides have led to defeat in infection removal.

The purpose of this study was to evaluate the effect of tigecycline, azithromycin, colistin, and other selected antibiotics against ESBL/carbapenemase-producing *K. pneumoniae*. The reason for designing the present study includes the increasing problem of XDR *K. pneumoniae* in hospitals, the spread of such strains associated with high mortality rates, limited treatment options, and attempting to make use of these new drug delivery systems.

2. Methods

2.1. Bacterial strains

Ninety-one isolates were identified as *K. pneumoniae*, using standard phenotypic microbiological tests and API 20E commercial strips (bioMérieux, France). These ninety-one non-duplicate *K. pneumoniae* were selected from COVID-19 patients hospitalized in ICUs with suspected VAP cases that were positive for BAL fluid and endotracheal aspirate (ETA) by semi-quantitative culture. ETA semi-quantitative cultures were moderate or heavy growth. VAP suspected patients were detected via at least two of the following criteria: temperature over 38° C or under 36°C, purulent respiratory secretions, leukocyte count of over 10,000/mm^3, or leukopenia under 4,000/mm^3. Furthermore, diagnosing VAP, requires a high clinical suspicion combined with bedside examination, radiographic examination, and microbiologic analysis of respiratory secretions. All isolates were confirmed to be *K. pneumoniae* using 16S *rRNA* analysis after PCR amplification with the universal primers (27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT). Finally, from 91 *K. pneumoniae* isolated twenty-seven ESBL/carbapenemase-producing *K. pneumoniae* strains were collected (September 2021 to February 2022). Isolates were stored at −20 °C in Tryptic Soy Broth (TSB) containing 20% glycerol until further studies.

2.2. Antibiotic Susceptibility Testing

Antimicrobial susceptibility test (AST) was performed by the disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI; 2022) (10). Antibiotic disks include levofloxacin (LEV) (5 µg), azithromycin (AZT) (15 µg), cefotaxime (CTX) (30 µg), cefotaxime/clavulanate (30/10 µg), ceftazidime (30 µg), ceftazidime/clavulanate (30/10 µg) (30 µg), amikacin (AN) (30 µg), gentamicin (GN) (10 µg), cefepime (FEP) (30 µg), imipenem (IMP) (5 µg), meropenem (MEN) (5 µg), piperacillin/tazobactam (PTZ) (100/10 µg), piperacillin (PIP), ciprofloxacin (CP) (5 µg), Trimethoprim- sulfamethoxazol (SXT) (25µg), tobramycin (TOB) (10µg), and cefoxitin (FOX) (10µg). The MICs of colistin sulfate (Sigma–Aldrich, 122 Darmstadt, Germany) and tigecycline (European Pharmacopoeia, Strasbourg, France) were determined using the broth microdilution method, and the results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint recommendations. Azithromycin were tested for azithromycin susceptibility by disk diffusion and broth microdilution in Mueller-Hinton media, according to the CLSI 2022 (10). Azithromycin is considered anti-gram-positive antibiotic, and no CLSI or EUCAST breakpoints are prepared, for Enterobacterales, except for *Salmonella Typhi* and *Shigella* spp. In our experiments, twofold serial dilutions ranging from 64 - 0.5 µg/mL for azithromycin were prepared using cation-adjusted Mueller-Hinton broth (CAMHB) (11).

Stock solutions were prepared on the same day of inoculation, freshly. *Escherichia coli* ATCC 25922, and *K. pneumoniae* ATCC 700603 were included in each run as a control. The multidrug-resistant (MDR), XDR, and non-MDR according to the international expert proposal for interim standards guidelines (12), as follows: XDR was defined as acquired resistance to ≥ 1 agent in all but ≤ 2 categories, MDR as resistance to ≥ 1 agent in ≥ 3 antimicrobial categories and, non-MDR as resistance to 0-2 antimicrobial categories.

2.3. Phenotypic screening of ESBL, AmpC betalactamase, and Carbapenemase

Producer-*K. pneumonia*

Based on guidelines of the CLSI 2022 combined disk method was used for screening ESBL production among *K. pneumonia*. Briefly, susceptibility to cefotaxime (30 µg), cefotaxime/clavulanate (30/10 µg), ceftazidime (30 µg), and ceftazidime/clavulanate (30/10 µg) (Mast Co., UK) was determined on Muller-Hinton agar (Merck Co, Germany). The ESBL-producing test result was defined as an increase in the diameter of the area around the ceftazidime/clavulanate and cefotaxime/clavulanate disks by ≥ 5 mm compared to the disks without clavulanic acid (Provided that the bacterial isolate is resistant to agent when tested alone) (10). *E. coli* ATCC 35218 was used as control strain. A cefoxitin disk (30 µg) was used to screen AmpC-producing isolates. A double-disk synergy test was performed with cefoxitin-bronic acid to determine AmpC production (13). To screen carbapenemase-producing isolates the Modified Hodge Test (MHT) was performed. *K. pneumoniae* ATCC BAA-1705 and BAA-1706 were used as MHT-positive and negative controls (10).

2.4. Detection of ESBL, AmpC, and Carbapenemase-Related Genes

The PCR was performed to detect genes encoding AmpC (bla_{ACC}, bla_{DHA}, bla_{EBC}, bla_{FOX}, bla_{MOX}, and bla_{CIT}), ESBLs (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}), and carbapenemase (bla_{IMP}, bla_{VIM}, bla_{NDM1}, bla_{KPC}, *and* $bla_{OXA-48-like}$). All primer sequences used are listed in Table 1. The products were separated by electrophoresis in 1% agarose gel with 1×TBE (Tris/borate/EDTA) buffer, stained with safe stain load dye (CinnaGen Co., Tehran, Iran), and visualized under ultraviolet illumination.

2.5. Detection of *mcr-1-5* genes

The PCR testing was conducted for plasmid-mediated colistin resistance detection associated with *mcr-1-5*(14).

2.6. Multilocus Sequence Typing (MLST)

Strain typing among four colistin-resistant *K. pneumoniae* isolates was examined by MLST,

following the protocol described on the Pasteur MLST site (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html). All primer sequences used in MLST are listed in Table 2.

2.7. Biofilm Formation Assays

The biofilm formation capacity of all strains was determined by the crystal violet staining method described previously (15). Briefly, biofilm formation was conducted by growing bacteria isolates in a Cell culture plate (96 well). Bacterial suspension adjusted to 0.5 McFarland turbidity, and 200-µL of suspension was inoculated in each well, and incubated at 37°C for 48h. Then, the plates were washed three times with PBS, and each well was stained with 200 µL of 1% crystal violet for 20 min at ambient temperature. The plates were again washed three times to remove excess stains. The crystal violet attached to the adherent bacteria was solubilized with 180 µl of 33 % glacial acetic acid and the absorbance was read at OD570. Un-inoculated LB medium was used as a negative control, while the reference strain ATCC 700603 was selected as positive a control). Biofilm formation was classified into four different groups using the following formulas: If OD < ODc, the biofilm was not formed (negative), If ODc < OD < 2xODc, the biofilm was weak, if 2xODc < OD < 4xODc, the biofilm was moderate. If 4xODc < OD, the biofilm was strong .

2.8. Detection of virulence genes

In this study, HvKp could be defined as: positive capsular types K1, K2, positive siderophore genes ≥2 (*entB, iutA, iucA, Irp2*), or ≥۲ positive capsule-regulating genes (*magA, wcaG, rmpA*) and positive adhesions (*mrkA, mrkD, fimH*). Non-hvKp is termed as CKp (classic *K. pneumonia*) (16).

The *k. pneumoniae* isolates were screened by PCR for the following virulence genes: Type 1 fimbrin Dmannose specific adhesion (*fimH*), The type 3 fimbrial Adhesion (*mrkD*), enterobactin (*entB*), aerobactin siderophore biosynthesis (*iucA*) and its captor

(*iutA*), Yersiniabactin high- pathogenicity island (*irp-2*), capsular polysaccharide (*magA, wcaG*), hyper capsule: regulator of mucoid phenotype (*rmpA)* and type 3 fimbriae (*mrkA*). The primers used to identify these genes were designed using Allele ID 6 software and BLAST using the program on the NCBI website. All primers sequences used are listed in Table 3.

Table 1 Primers of *K. pneumoniae* genes for encoding AmpC, ESBLs and carbapenemase

Target	Sequence (5' to 3')	Size(bp)	References
KPC	F: CGTCTAGTTCTGCTGTCTTG R: GCGGCGTTATCACTGTATTG	383	In study
OXA-48	F: GGCGTAGTTGTGCTCTGG R: TATAGTCACCATTGGCTTCGG	487	In study
SHV	F: ATCCACTATCGCCAGCAG F: CCTCATTCAGTTCCGTTTCC	232	In study
CTX-M	R: AGGAAGTGTGCCGCTGTATG F: CTGTCGCCCAATGCTTTACC	552	In study
TEM-1	R: TCGCCGCATACACTATTCTC F: AACTTTATCCGCCTCCATCC	373	In study
NDM-1	F: ATACCGCCTGGACCGATGAC R: GAGATTGCCGAGCGACTTGG	395	In study
VIM	F: TGTCGCAAGTCCGTTAGC R: GCAGCACCAGGATAGAAGAG	480	In study
IMP	F: TTAGCGGAGTTAGTTATTGGC R: TTAGTTACTTGGCTGTGATGG	335	In study
MOX	F: GCT GCT CAA GGA GCA CAG GAT R: CAC ATT GAC ATA GGT GTG GTG C	520	(29)
FOX	F: AAC ATG GGG TAT CAG GGA GAT G R: CAA AGC GCG TAA CCG GAT TGG	190	(29)
CIT	F: TGG CCA GAA CTG ACA GGC AAA R: TTT CTC CTG AAC GTG GCT GGC	462	(29)
DHA	F: AAC TTT CAC AGG TGT GCT GGGT R: CCG TAC GCA TAC TGG CTT TGC	405	(29)
ACC	F: AAC AGC CTC AGC AGC CGG TTA R: TTC GCC GCA ATC ATC CCT AGC	346	(29)
EBC	F: TCG GTA AAG CCG ATG TTG CGG R: CTT CCA CTG CGG CTG CCA GTT	302	(29)

Table 2 Primers used for identification of Strain Typing (MLST) of *K. pneumoniae* (29)

Gene name	Sequences (5' to 3' end)	Amplicon size
gapA	F: TGAAATATGACTCCACTCACGG R: CTTCAGAAGCGGCTTTGATGGCTT	662
infB	F: CTCGCTGCTGGACTATATTCG R: CGCTTTCAGCTCAAGAACTTC	462
mdh	F: CCCAACTCGCTTCAGGTTCAG R: CCGTTTTTCCCCAGCAGCAG	756
pgi	F: GAGAAAAACCTGCCTGTACTGCTGGC R: CGCGCCACGCTTTATAGCGGTTAAT	718
phoE	F: ACCTACCGCAACACCGACTTCTTCGG R: TGATCAGAACTGGTAGGTGAT	602
rpoB	F: GGCGAAATGGCWGAGAACCA R: GAGTCTTCGAAGTTGTAACC	1075
wzi	F: GTGCCGCGAGCGCTTTCTATCTTGGTATTCC R: GAGAGCCACTGGTTCCAGAAYTTSACCGC	580

Table 3 Primer use in PCR for virulent genes and capsular typing of *K. pneumoniae*

Target gene	Primer sequence (5´→3´)	Amplicon size
fimH	F: GCTGCTGCTGGGCTGGTC R: GGTCGGGAACGGGTAAGAGG	292 bp
mrkA	F: AATGTAGGCGGCGGTCAG R: CTCTCCACCGATAACGCCA	351 bp
mrkD	F: CTGAGTGAAACGGGATATGC R: AGCGGTATGGTGATGTAGC	224 bp
magA	F: CATTGCCGCTACTACAGGAG R: AGTGAACGAATTGATGCTTGG	239 bp
entB	F: GCATCGGTGGCGGTGGTC R: CGGCGAACAAGGTCAACTGG	439 bp
Irp2	F: GCAACGGCGGGCATAGTC R: GCGAGGTCTGGCTACAATGG	320 bp
wcaG	F: AGCAACCGATTAGTGAGTCC R: TCAACGCCAGTGCCTACG	402 bp
iutA	F: GGGAAAGGCTTCTCTGCCAT R: TTATTCGCCACCACGCTCTT	920bp
iucA	F: AATCAATGGCTATTCCCGCTG R:CGCTTCACTTCTTTCACTGACAGG	239bp
rmpA	F: CATAAGAGTATTGGTTGACAG R: CTTGCATGAGCCATCTTTCA	461bp
K1	F: GTAGGTATTGCAAGCCATGC R: GCCCAGGTTAATGAATCCGT	1047
K2wzy	F: GACCCGATATTCATACTTGACAGAG R: CCTGAAGTAAAATCGTAAATAGATGGC	641
K2	F: CAACCATGGTGGTCGATTAG R: TGGTAGCCATATCCCTTTGG	531

2.9. Statistical Analysis

Descriptive statistics were used to measure the characteristics of the study. Pearson chi-square test was used to determine significant differences between proportion. P values of <0.05 were considered significant. Statistical analysis was performed by using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Antimicrobial susceptibility

By phenotypic detection tests and molecular methods 27/91 (29.6%) of *K. pneumoniae* isolated from hospitalized patients in ICUs, were classified as ESBL/carbapenemase-producing *K. pneumoniae* strains that harbored at least 1 of the carbapenemase/ESBL-related genes.

In ninety-one *K. pneumoniae*, ESBL-associated genes (19.7% bla_{TEM}, 29.6% bla_{SHV}, and 19.7% $bla_{\text{CTX-M}}$) were detected. Also, carbapenemase-related genes were detected in 17.5% of isolates ($bla_{\text{OXA-48-like}}$ 15.4%, and bla_{NDM1} 2.1%). Among 27 beta-lactamase producing *K. pneumoniae*, ESBL associated genes (18 (66.7%) bla_{TEM}, 27 (100%) bla_{SHV}, and 18 (66.7%) $bla_{CTX\text{-}M}$) and carbapenemase-related genes (16 (59.3%)) were detected. The prevalence rates of these genes were $bla_{OXA\text{-}48\text{-}like}$ 14(51.9%), and bla_{NDM1} 2 (7.4%) in carbapenem-resistant *K. pneumoniae* (CRKP). The genes of bla_{IMP}, bla_{VIM}, and bla_{KPC} were not detected in isolates. Also, the AmpC-associated genes were not detected in any of the strains.

Based on the CLSI breakpoint and susceptibility testing results, from twenty-seven ESBL/carbapenemase-producing *K. pneumoniae* strains, 16 (59.3%) and 11 (40.7%) isolated strains were categorized as MDR and XDR strains respectively (Table 4). The MICs range of ESBL/CRKP isolates against tigecycline and colistin was 0.25–0.5 and 2–16 mg/L, respectively. Tigecycline was sensitive against all ESBL/CRKP isolates. The highest resistance rate in this study was against azithromycin (100%), and ceftazidime (85.18%)

followed by cefotaxime (92.5%) (Fig1).

Through the broth microdilution test, it was revealed that all isolates were highly sensitive to tigecycline and colistin (100% and 85.2%) (Table4). Another antibiotic with higher sensitivity was amikacin (44.4%). Phenotypic ESBL detection tests indicated that 27 (100%) *K. pneumoniae* isolates were ESBL producers, and they were all sensitive to tigecycline. *Mcr-1-5* genes were not detected in *K. pneumoniae* isolates in the current study.

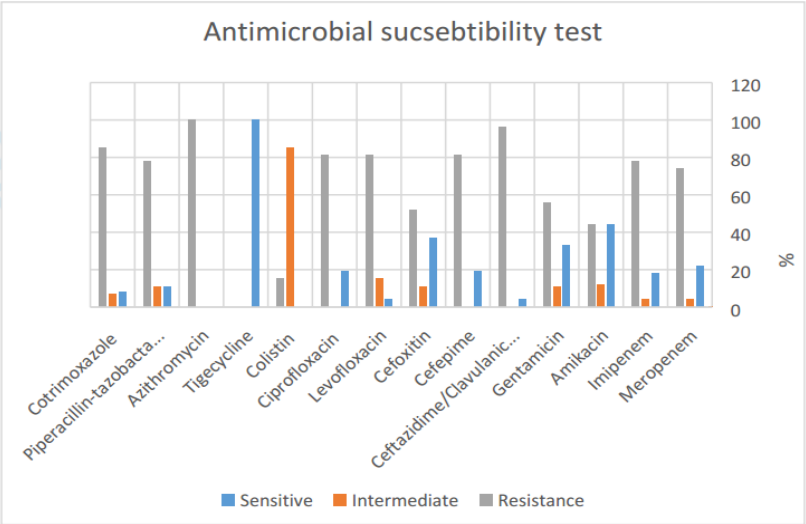

Fig.1 Diagram of the results of antibiotics susceptibility test

3.2. Molecular Typing

MLST analysis of four colistin-resistant *K. pneumoniae* revealed different STs and their STs were as follows: ST3500, ST273, and 2 cases of ST2558.

3.3. Assessment of biofilm formation capacity

All 27 selected *K. pneumoniae* isolates were determined to develop biofilm, 12 (44.44%) formed fully established biofilms, 9 (33.33 %) were categorized as moderately biofilm-producing, and 6 (22.22 %) formed weak biofilms.

3.4. Assessment of virulence factors

In general, nine of the 10 screened virulence factors (*fimH, irp2, iutA, mrkD, mrkA, wcaG, magA, rmpA, and entB*) except *iucA* were identified in the 27 *K. pneumonia*e isolates. All *K.*

pneumoniae isolates carried at least one biofilm-related gene.

Molecular distribution of virulence genes revealed that 92.59%, 92.59%, 81.48%, 88.8%, 40.74%, 22.22%, 18.5%, 14.81% and 33.33% of the ESBL/carbapenemase producer *K. pneumoniae* isolates carried *entB, mrkD, fimH, Irp2, wcaG, mrkA, rmpA, iutA* and *magA* genes, respectively (Fig 2). But *iucA* gene was not present in any of the isolates. The number of positive virulence genes determinants varied from three to eight genes in any isolate. Different percentages of fimbriae genes were identified, *fimH* gene was detected in 81.48% of isolates, but only 22.22% of the isolates were positive for *mrkA* gene.

3.5. The correlation between biofilm formation and antibiotic resistance phenotypes

The majority of strong biofilm-forming *K. pneumoniae* isolates were XDR. Only 25% of MDR isolates were strong biofilm producers, whereas 73% of XDR isolates form strong biofilms (Fig 3 and Fig 4). It should be noted that the majority of XDR isolates carried both *magA* and *mrkA* virulence genes. Most of the XDR isolates were from the more virulent serotype of K1. In K1 isolates, the *magA* gene is essential for the formation of the exopolysaccharide, a process that can be enhanced by *rmpA*. In the current study, only one isolate was detected as hvkp (Table 4).

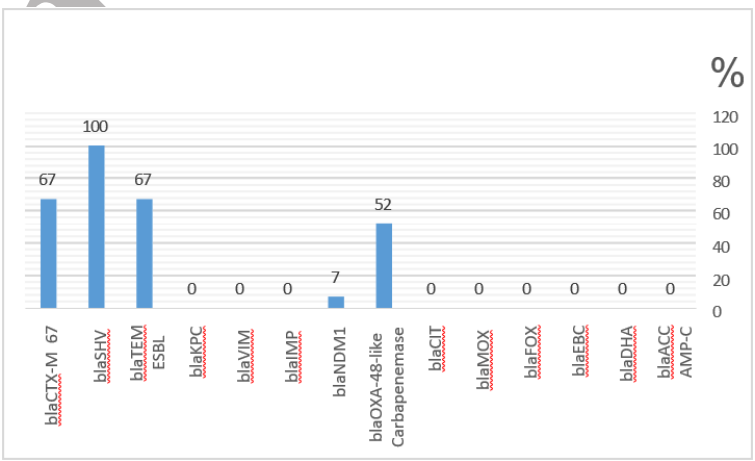

Fig.2 Diagram of the results of ESBL, AmpC, and Carbapenemase related genes

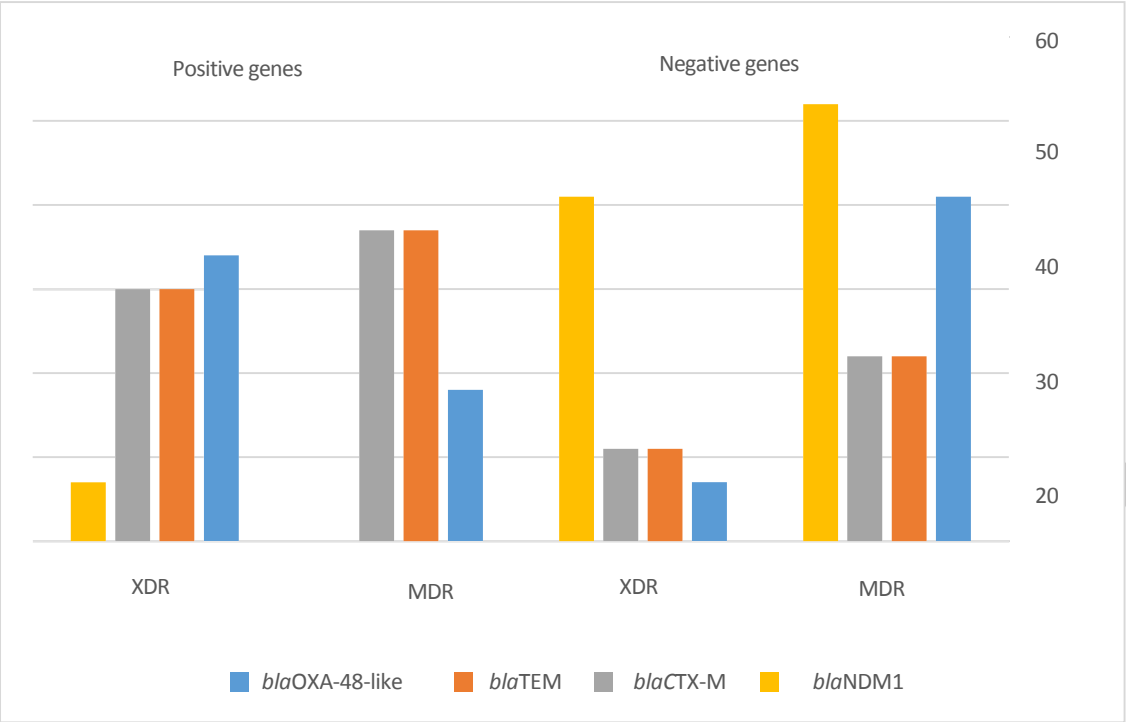

Fig.3 Comparative diagram of the results of antibiotics susceptibility test (MDR/XDR) and genes distribution

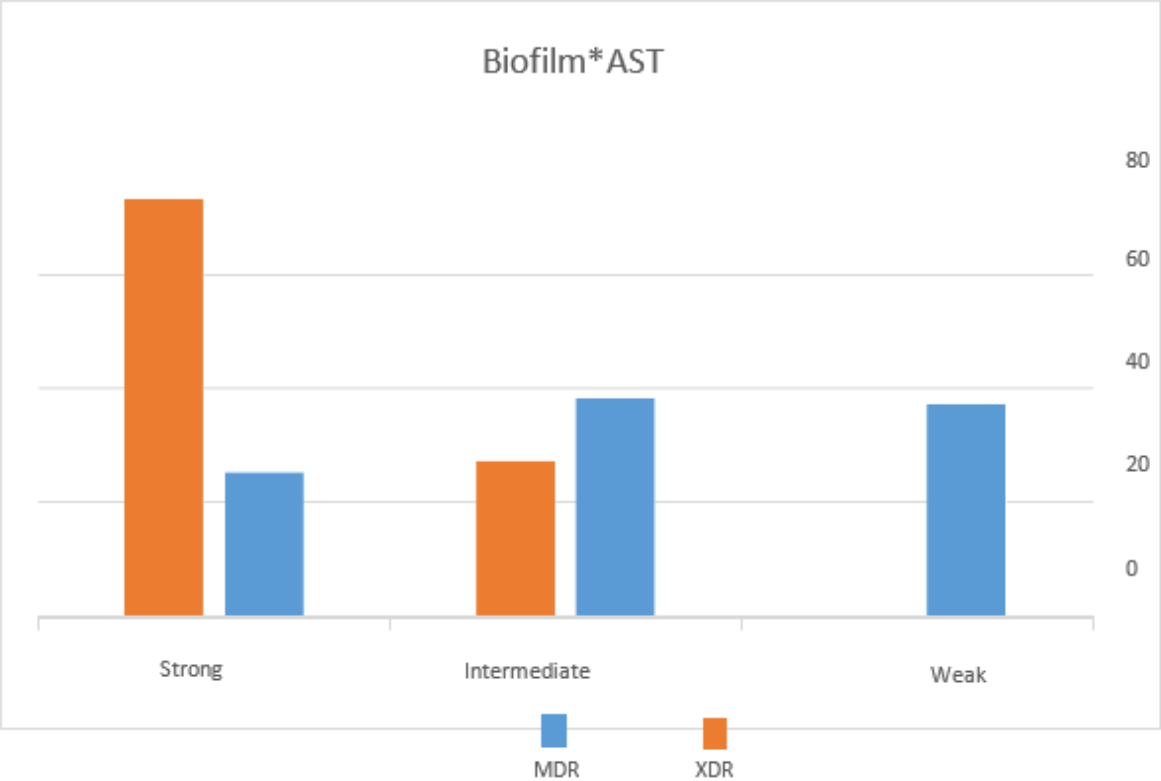

Fig.4 Diagram of biofilm production and AST and distribution of *fimH* and *magA* genes

Table 4 Antibiotic resistance profiles and MICs of tigecycline and colistin of twenty-seven ESBL /CRKP *Klebsiella pneumoniae* isolates.

	Type of pathogens; resistance characteristic																		
			MIC (mg/L)					AST		CPS biosynthesis			Adhesion			Sider ophores				
Isolates	ESBL genotype	Carbapenemase genotype	Colistin	Tigecycline	Azitromycinn	MDR/XDR	Biofilm		Capsule serotype	*rmpA*	*magA*	*wcaG*	*mrkD*	*mrkA*	*fimH*	*entB*	*iutA*	*iucA*	*Irp2*	Patient characteristics
1	SHV, CTX-M, TEM	OXA-48	0.5	0.5	≥64	MDR	Interm ediate	PTZ, LEV, FEP, IPM, MEN, PTZ, CAZ, CZA, AZ, PIP, CTX, FOX	K non-T				+		+	+			+	68-year-old male with history of cancer
2	SHV, CTX-M, TEM	-	0.5	0.5	≥64	MDR	Interm ediate	FEP, PTZ, CRO, SXT, AZ, LEV, PIP, CTX	K2			+	+		+	+			+	69-year-old male
3	SHV	-	0.5	0.25	≥64	MDR	Strong	LEV, AZ, CZA, CAZ, SXT, PIP, CTX	K2	+			+		+	+	+		+	65-year-old female
4*	SHV, CTX-M, TEM	-	16	0.5	16	XDR	Strong	CZA, AN, GM, TOB, CTX, FEP, MEN, AZ, PTZ, CAZ, CP, CRO, SXT, CL	K1	+	+	+	+	+	+	+			+	71-year-old female with diabetes
5	SHV, CTX-M, TEM	OXA-48	0.5	0.5	≥64	XDR	Strong	LEV, AZ, CZA, AN, GM, FEP, TOB, IPM, MEN, PTZ, CRO, SXT, CAZ, CP, CTX, FOX	K1		+	+			+	+			+	82-year-old man with diabetes mellitus
6	SHV, CTX-M, TEM	-	16	0.25	32	MDR	Strong	CL, LEV, CZA, AZ, CTX, FOX	K1	+	+		+		+	+			+	74-year-old male with kidney and urinary tract diseases
7	SHV,TEM	-	0.5	0.5	≥64	MDR	Weak	CTX, AZ, CZA, FEP, IPM, MEN, PTZ, CAZ, CP, CRO	K non-T				+							68-year-old male
8	SHV, CTX-M, TEM	-	0.5	0.5	≥64	MDR	Strong	CTX, AZ, CZA, FEP, IPM, MEN, PTZ, CAZ, CP, SXT	K1				+		+	+			+	69-year-old female with diabetes
9	SHV	OXA-48	0.5	0.25	≥64	MDR	Interm ediate	CZA, FEP, IPM, MEN, PTZ, CAZ, CP, SXT,PIP	K1	+			+		+	+	+		+	68-year-old male
10	SHV, CTX-M, TEM	-	0.5	0.5	≥64	MDR	Weak	AZ, CAZ, PIP, CRO, SXT, CTX	K non-T				+			+			+	65-year-old male
11	SHV, TEM	OXA-48	0.5	0.5	≥64	XDR	Strong	CL, CZA, AN, GM, FEP,CTX, MEN, PTZ, CAZ, CP, SXT, FOX, TOB, LEV, AZ, PIP	K2			+	+	+	+	+			+	77 year-old male with diabetes, chronic renal failure
12	SHV, CTX-M, TEM	OXA-48	16	0.25	32	MDR	Interm ediate	AZ, FEP, IPM, MEN, PIP, PTZ,CTX, GM, CAZ, CP	K non-T				+		+	+			+	58-year-old male
13	SHV, CTX-M, TEM	OXA-48	0.5	0.5	≥64	XDR	Strong	LEV, AZ, CZA, AN, GM, FEP, TOB, IPM, MEN, PTZ, CRO,	K1		+		+		+	+			+	64-year-old male with diabetes

								SXT, CAZ, CP, CTX, FOX											
14	SHV, CTX-M, TEM	NDM-1	0.5	0.25	≥64	MDR	Intermediate	CTX, FEP, CAZ,FOX	K2			+	+		+	+			+	71-year-old male with diabetes
15	SHV, CTX-M	OXA-48	0.5	0.5	≥64	XDR	Strong	LEV, AZ, CZA, AN, FOX, FEP, IPM, GM, CTX, MEN, PTZ, CAZ, CP, CRO, SXT	K2			+	+		+	+			+	68-year-old male with kidney and urinary tract diseases
16	SHV	-	0.5	0.5	≥64	MDR	Weak	AZ, CZA, AN, FEP, IPM, MEN, PTZ, CAZ, CP, CTX, SXT	K non-T				+			+				68-year-old male
17	SHV, TEM, CTX-M	OXA-48	0.5	0.25	≥64	XDR	Strong	LEV, AZ, CZA, AN, GM, FEP, IPM, MEN, PTZ, CAZ, CTX, GM, FEP, IPM, MEN, PTZ, CAZ, CP, CRO, SXT	K1		+		+		+	+			+	79-year-old female
18	SHV, CTX-M	OXA-48	2	0.5	≥64	MDR	Weak	CTX, AZ, CZA, GM, FEP, IPM, MEN, PTZ, CAZ	K non-T				+		+	+			+	66-year-old male
19	SHV, TEM	NDM-1	0.5	0.5	≥64	MDR	Intermediate	CZA, AN, GM, FEP, LEV, IPM, AZ, PTZ, CAZ, CP, SXT, FOX	K2			+	+		+				+	78-year-old male
20	SHV, CTX-M, TEM	OXA-48	0.5	0.25	≥64	MDR	Weak	LEV, AZ, FEP, CZA, IPM, MEN, PTZ, CAZ, CP, CTX, SXT	K non-T				+			+			+	53-year-old female
21	SHV, CTX-M	OXA-48	8	0.25	64	XDR	Intermediate	CL, LEV, AZ, AN, CZA, FEP, IPM, CP, CRO, SXT, PTZ, CAZ, FOX, TOB, CTX	K1		+	+	+	+	+	+			+	52 year-old male solid organ transplant recipient
22	SHV, CTX-M, TE	OXA-48	1	0.5	≥64	XDR	Intermediate	LEV, AZ, CZA, AN, GM, FEP, IPM, CTX MEN, PTZ, CAZ, CP, CRO, SXT, FOX	K non-T			+	+	+	+	+	+		+	69-year-old female with diabetes
23	SHV, CTX-M	OXA-48	2	0.5	≥64	XDR	Strong	LEV, AZ, CZA, GM, FEP, IPM, MEN, CP, SXT, CRO, FOX, CAZ, CTX	K1		+	+	+		+	+			+	51-year-old female with history of breast cancer
24	SHV, CTX-M, TEM	-	0.5	0.5	≥64	MDR	Weak	AZ, CRO, PTZ, CTX	K non-T				+			+				72-year-old female with diabetes
25	SHV, CTX-M, TEM	OXA-48	2	0.5	≥64	XDR	Strong	LEV, AZ, CZA, FEP, IPM, MEN, PTZ, CAZ, CP, CRO, SXT, AM, FOX, GM, PIP, CTX	K1	+	+		+		+	+			+	80-year-old male
26	SHV, CTX-M, TEM		2	0.5	≥64	XDR	Strong	LEV, AZ, CZA, AN, GM, FEP, IPM, CTX, MEN, PTZ, CAZ, CP, SXT, CRO, PIP, FOX	K1		+	+		+	+	+			+	69-year-old male
27	SHV		1	0.5	≥64	MDR	Intermediate	AZ, CZA, AN, CAZ, CP, CTX,FEP, IPM, MEN, PTZ, SXT	K non-T				+		+	+	+		+	47-year-old female with history of -breast cancer
	* hypervirulent *K. pneumoniae* (hvkp) Disk diffusion (mm) EUCAST European Committee on Antimicrobial Susceptibility Testing S ≥ 18, Tigesycline																		

3.6. Association between the presence of virulence genes and biofilm formation

According to PCR results for detecting virulence genes, the presence of the *fimH* gene was not detected among five weak biofilm producers with K Non-Type. Moreover, nine of the strong biofilm producers had the *magA* gene, while one of the intermediate biofilm producers was positive for the presence of this gene. The *entB* and *mrkD* virulence genes were positive in the most of isolates. The presence of the *irp2* gene was confirmed among strong, moderate biofilm-producers, and 3 (50%) weak biofilm-producers.

4. Discussion

The aim of the current study was to provide a point of reflection on the risk of ESBL/CRKP colonization and hospital-acquired infection in hospitalized patients in ICUs. Among the isolates of *K. pneumoniae*, almost one-third was producers of ESBL and Carbapenem-resistant *K. pneumoniae* (CRKP). In this study, 50% and 56.2% of ESBL/CRKP isolates were resistant to meropenem and imipenem, respectively. In line with previous studies (17), tigecycline was the most effective antimicrobial agent against these isolates. Other antibiotics in our study with higher sensitivities were colistin (85.2%) and amikacin (44.4%) respectively. The results of this study are consistent with the results of previous studies, which investigated the sensitivity of tigecycline (88.6% susceptibility) and colistin (73.9%) against carbapenem-resistant Enterobacterales (CRE) (18). Based on recent reports, it was found that tigecycline is one of the most active antimicrobial agents against gram-negative and gram-positive isolates including drug-resistant pathogens (19). Tigecycline is still the best choice for MDR-CRE strains, because of their high sensitivity to this agent (19). Among 27 *K. pneumoniae* isolates, 14 (51.8%) isolates were positive for the $bla_{\text{OXA-48}}$–type gene, which did not demonstrate the co-existence of other carbapenemases except for $bla_{\text{NDM-1}}$. This is reflective of a high prevalence of OXA-48-positive *K. pneumoniae* in this study. *NDM-1* was the second most frequent carbapenemase by 2 (7.4%) isolates. Similarly, $bla_{\text{OXA-}}$

$_{48}$ gene has recently been reported in the Middle East and is considered to be the most common carbapenemase in Middle-Eastern countries (20). The *bla*$_{NDM1}$ gene was reported first in India and was recently reported in Europe, North America, Asia, and Australia (20). The simultaneity of *bla*$_{NDM}$ and *bla*$_{OXA-48}$ genes among *K. pneumoniae* has also been identified in several countries. The high prevalence of *bla*$_{OXA-48}$ and *bla*$_{NDM1}$ genotypes may be explained by the fact that Iran takes a large number of immigrants or visitors from *bla*$_{OXA-48}$ and *bla*$_{NDM}$ high prevalence countries. Moreover, this study also revealed that 3 types of enzymes (VIM, IPM, and KPC) were not significant types of carbapenemases. The results are consistent with research conducted by Gheitani *et al*., which is showed that the prevalence rates of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{KPC}$ were 4 (2.18%), 1 (0.5%), and 0%, respectively (21). Results of the current study, showed a high proportion of SHV, CTX-M, and TEM enzymes among ESBL-producing *K. pneumoniae* strains in ICUs hospitalized patients due to COVID-19. Our data were consistent with other studies conducted in Iran and other parts of the world (22). It seems that isolates harbored *bla*$_{SHV}$ as the predominant genotype in this study. The situation related to ESBL production in Iran is very different, ranging from 9.8% to 75.7% (23). In this study, no genes related to *bla*AmpC were detected. In contrast, perhaps the PCR results in our study were inconsistent with some surveys conducted in other parts of the world, due to genetic differences in causative strains, the use of antibiotics, and access to broad-spectrum and new antibiotics (24). In this study, the MDR/XDR isolates harbored ESBL/CRKP genes rendering most antibiotic mono-therapies ineffective.

Another study conducted in 18 European countries, indicated that the susceptibility rate of tigecycline to carbapenem resistance Enterobacterales is 88.6%, which is in line with our findings (25).

Colistin, and some aminoglycosides still show favorable in vitro activities against carbapenem-resistant Enterobacterales. It can be suggested that against MDR/XDR isolates

harbored ESBL/CRKP genes and *mcr* genes use of combinatorial pharmacodynamics of colistin and tigecycline is more effective (26). Combine therapy, preventing the increased resistance to colistin, and the ability for decreasing colistin and tigecycline MICs (27). Increasing antibiotic resistance among biofilm-producing isolates raises serious concerns about limited treatment options in hospitals. Based on the surveys, substantial actions and the introduction of new strategies are needed to control *K. pneumoniae* biofilm-related infections. In this work, it was revealed that most XDR isolates tended to develop stronger biofilms compared to MDR isolates, and it is suggested a direct relationship between XDR and biofilm formation capacity. Another study indicated that, in the KPC-positive group, the *irp2, mrkD*, and *fimH* virulence genes had a higher frequency than in the KPC-negative group (28). Therefore, the presence of genes of *entB, magA, Irp2*, *fimH*, and *mrkD* which are found in our survey, illustrates the importance of evaluating these virulence factors. It should be noted that the differences in results could be due to differences in the study population.

The results of this study demonstrated the prevalence of infections caused by β-lactamase-producing *K. pneumoniae*, which are biofilm producers in ICUs. In the current study, all isolates produced strong and moderate biofilm. The results indicated that strong and moderate biofilm formation isolates need to address new categories of antibiotics. The effective antimicrobial activity of tigecycline against bacteria that produce these enzymes may be efficient in faster and better treating patients who are hospitalized. The monitoring and control of hospital-acquired infections should be considered, to reduce the spread of MDR/XDR bacteria. These include surveillance systems to notice changes in drug resistance profile and etiology, setting experimental treatment guidelines based on the profiles and proper instruction of healthcare workers regarding sanitation. Future studies should include more complex microbial communities residing in the hospitals. Also, the field of study using new

antibiotics should be addressed.

Declarations

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Author contribution

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by S.R, M.B, F.N, and M.KH. The first draft of the manuscript was written by S.R and M.B, and all authors commented on previous versions of the manuscript. H.E contributed to the manuscript's final version and supervised the research process. S.R, M.B, H.S.S and F.N prepared figures and tables. All authors read and approved the final manuscript. All authors reviewed the manuscript**.**

Competing interests

The authors declare that they have no competing interests

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Ethics approval and consent to participate

The current study was performed by approval of the ethics committee of Qazvin Medical University with approval number IR.QUMS.REC.1400.166. In addition, the committee approved the utilization of human samples. We confirm that written informed consent to participate was obtained from all of the participants in our study. We acquired permissions and/or licenses to access the clinical patient data used in our research from Qazvin University of Medical Sciences. Hospitals provided the clinical samples. Also, it should be noted that biological samples are handled by the authors in the present study. The adopted methods for handling human samples were carried out in accordance with relevant

guidelines and regulations provided in the Declaration of Helsinki. The research protocol was approved by the Research Ethics Committee at the Qazvin Medical University, Iran.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

List of abbreviations

ESBL: Extended-spectrum-β-lactamase, CRE: Carbapenem-Resistant Enterobacterales, CRKP: Carbapenemase-producing *Klebsiella pneumonia*, BAL: Bronchoalveolar lavage, TSA: Trypticase soy agar, KPC: *K. pneumoniae* Carbapenemase, MDR: Multi-drug resistance, XDR: Extensively-drug resistance, ICUs: Intensive care unites

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Fig.1 Diagram of the results of antibiotics susceptibility test

Fig.2 Diagram of the results of ESBL, AmpC, and Carbapenemase related genes

Fig.3 Comparative diagram of the results of antibiotics susceptibility test (MDR/XDR) and genes distribution

Fig.4 Diagram of biofilm production and AST and distribution of *fimH* and *magA* genes