Original Article

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

Sara Rahimi[1,2], Mohadeseh Khakpour[1,2], Mehdi Bakht[1,2], Hasan Ehteram[3], Hediyeh Saghi Sarabi[1], Farhad Nikkhahi[1*]

1. *Medical Microbiology Research Center, Qazvin University of Medical Sciences, Qazvin, Iran.*
2. *Student research committee, Qazvin University of Medical Sciences, Qazvin, Iran.*
3. *Department of pathology. Kashan University of medical sciences Kashan. Iran.*

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Corresponding Author's E-Mail:
FARHADNIKKHAHI@gmail.com

ABSTRACT

Pneumonia caused by Klebsiella pneumoniae (K. pneumoniae) is regarded as one of the most prevalent etiologies of nosocomial infections. The objective of this study was 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 patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. The present study investigates the activity of tigecycline, azithromycin, and colistin against ESBL/carbapenemase-producing K. pneumoniae. The investigation encompasses the phenotypic and genotypic screening of ESBLs, AmpC beta-lactamases, and carbapenemase enzymes. Furthermore, an evaluation was conducted to ascertain the capacity of the biofilm to form. Consequently, the presence of virulence genes was identified through the implementation of a polymerase chain reaction (PCR) method. The utilization of phenotypic detection tests resulted in the categorization of 27 (29.6%) out of 91 K. pneumoniae isolates as ESBL/carbapenemase-producing K. pneumoniae strains. Furthermore, molecular methods revealed that all 27 K. pneumoniae isolates possessed at least one of the ESBL/carbapenemase-related genes. ESBL-associated genes were detected in 91 K. pneumoniae isolates, including 19.7% blaTEM, 29.6% blaSHV, and 19.7% blaCTX-M. Carbapenemase-related genes were identified in 17.5% of the isolates, including blaOXA-48-like (15.4%) and blaNDM1 (2.1%). The investigation revealed that all 27 of the isolates demonstrated the capacity to form biofilms. In this study, the prevalence of specific genes among ESBL/carbapenemase producer K. pneumoniae isolates was investigated. The genes analyzed included entB, mrkD, fimH, Irp2, wcaG, mrkA, rmpA, iutA, and magA. The results showed that 92.59%, 92.59%, 81.48%, 88.8%, 40.74%, 11.1%, 22.22%, 18.5%, 14.81%, and 33.33% of the isolates carried entB, mrkD, fimH, Irp2, wcaG, mrkA, rmpA, iutA, and magA genes, respectively. However, the iucA gene was not detected in any of the isolates examined. Tigecycline and colistin demonstrated higher efficacy against these isolates. Multilocus sequence typing (MLST) results for four colistin-resistant isolates revealed three distinct sequence types (ST): ST3500, ST273, and two cases of ST2558. The rapid emergence and subsequent dissemination of colistin-resistant and Beta-lactamase-producing K. pneumoniae has led to a worrisome global situation. 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 associated with high morbidity and mortality due to the limited number of antibiotic treatment options (1). Consequently, carbapenems have been regarded as effective treatment options for infections caused by ESBL-producing K. pneumoniae. Capsular serotypes K1 and K2 in K. pneumoniae strains have been identified as risk factors for liver abscess and complicated endophthalmitis (2). These serotypes are the most prevalent isolates from patients worldwide. Carbapenems are regarded as the most reliable last-resort treatment for bacterial infections due to their high efficacy against a wide range of bacterial species and their resilience to most beta-lactam resistance determinants (3). The carbapenems have been demonstrated to be safer than other last-line drugs, such as polymyxins. Consequently, the advent and rapid propagation of carbapenem resistance on all continents, regarded as the last-resort antibiotics for the treatment of ESBL-producing K. pneumoniae, has emerged as a pervasive public healthcare concern (4). The excessive utilization of carbapenems in healthcare facilities has resulted in a surge in carbapenem-resistant K. pneumoniae infections. The emergence of K. pneumoniae carbapenemase (KPC)-producing bacteria is a cause for concern. It has been established that there are several mechanisms that result in resistance to carbapenems. These mechanisms include the production of carbapenemase enzymes of classes A (KPC, GES, and others), B (mainly IMP, VIM, or NDM), and D (OXA-48) and related enzymes. For carbapenem-resistant isolates, treatment of infections is best managed with tigecycline, a glycylcycline derivative of minocycline, as a last resort (5). K. pneumoniae isolates classified as extensively drug resistant (XDR) are emerging in rapid succession due to the dissemination of resistance to aminoglycosides, fluoroquinolones, β-lactams, and carbapenems. Newly emerged XDR strains have evolved to become PDR by developing resistance to tigecycline and polymyxin antibiotics (6). The emergence of XDR and hypervirulent Klebsiella pneumoniae (XDR-hvKp) represents a novel challenge for patients in intensive care units. This strain of bacteria, classified as a superbug, has been identified as a primary contributor to nosocomial infections. It is imperative to emphasize the clinical management of beta-lactamase and biofilm producer K. pneumoniae infections, which have been identified as the next potential superbug (7). The prevalence of bacterial co-infection with coronavirus disease has been documented at varying rates; however, it has been reported to be as high as 50% in non-survivors (8). The present findings indicate that the most prevalent bacterial pathogens identified in secondary bacterial infections in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) include Mycoplasma pneumoniae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii, and Stenotrophomonas maltophila (9). These findings support the routine use of antibiotics in the management of co-infection associated with hospitalized patients in ICUs with severe acute respiratory syndrome (SARS) CoV-2, which makes them more exposed to nosocomial infections (9). The National Institute for Health and Care Excellence (NICE) has issued a recommendation for the administration of antibacterial treatment to patients with high risk of complications from untreated bacterial infections (8). The formation of biofilms and subsequent attachment to surfaces, in conjunction with the presence of capsular polysaccharides, have been identified as factors contributing to the failure of infection removal efforts. The objective of this study was to assess the efficacy of tigecycline, azithromycin, colistin, and other selected antibiotics against ESBL/carbapenemase-producing K. pneumoniae. The impetus for the present study stems from the mounting challenges posed by XDR K. pneumoniae in healthcare settings, the proliferation of such strains associated with elevated mortality rates, constrained treatment options, and the pursuit of innovative drug delivery systems.

2. Materials and Methods

2.1. Bacterial Strains

A total of 91 isolates were identified as K. pneumoniae using standard phenotypic microbiological tests and API 20E commercial strips (bioMérieux, France). A total of 91 non-duplicate K. pneumoniae isolates were selected from a cohort of patients diagnosed with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) who were admitted to intensive care units (ICUs) with suspected ventilator-associated pneumonia (VAP). These isolates were identified as positive for bronchoalveolar lavage (BAL) fluid and endotracheal aspirate (ETA) by semi-quantitative culture. According to the results of the ETA semi-quantitative cultures, the presence of moderate to heavy growth was observed. The suspicion of a patient with VAP was determined by the presence of at least two of the following criteria:

- Temperature greater than 38.0°C or less than 36.0°C
- Presence of purulent respiratory secretions
- Leukocyte count greater than 10,000/mm³ or less than 4,000/mm³ In addition, a high degree of clinical suspicion in conjunction with bedside examination, radiographic examination, and microbiologic analysis of respiratory secretions is imperative for the diagnosis of ventilator-associated pneumonia (VAP). The confirmation of all isolates as K. pneumoniae was achieved through the implementation of 16S rRNA analysis following the PCR amplification process utilizing universal primers (27F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT). A total of 27 ESBL/carbapenemase-producing K. pneumoniae strains were isolated from 91 K. pneumoniae isolates collected from September 2021 to February 2022. Isolates were stored at −20 °C in Tryptic Soy Broth (TSB) containing 20% glycerol until further studies were conducted.

2.2. Antibiotic Susceptibility Testing

The antimicrobial susceptibility test (AST) was performed by the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI; 2022) (10). Antibiotic disks contain the following medications: levofloxacin (LEV) at a concentration of 5 µg, azithromycin (AZT) at a concentration of 15 µg, cefotaxime (CTX) at a concentration of 30 µg, and cefotaxime/clavulanate at a concentration of 30/10 µg, respectively. The following antibiotics were utilized: ceftazidime (30 µg), ceftazidime/clavulanate (30/10 µg) (30 µg), amikacin (AN) (30 µg), and gentamicin (GN) (10 µg). The following antibiotics were utilized: cefepime (FEP) at a dosage of 30 µg, imipenem (IMP) at 5 µg, meropenem (MEM) at 5 µg, piperacillin/tazobactam (PZ) at 100/10 µg, and piperacillin (PIP). The following antibiotics were utilized: ciprofloxacin (CP) (5 µg), trimethoprim-sulfamethoxazol (SXT) (25 µg), tobramycin (TOB) (10 µg), and cefoxitin (FOX) (10 µg). The MICs of colistin sulfate (Sigma–Aldrich, 122 Darmstadt, Germany) and tigecycline (European Pharmacopoeia, Strasbourg, France) were determined using the broth microdilution method. The results were interpreted based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint recommendations. The testing of azithromycin for susceptibility was conducted in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines, employing disk diffusion and broth microdilution methods in Mueller-Hinton media (10). Azithromycin is classified as an antibiotic that acts against gram-positive bacteria. No Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints have been established for Enterobacterales, with the exception of Salmonella Typhi and Shigella species. In the present study, twofold serial dilutions ranging from 64 to 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*

According to the Clinical and Laboratory Standards Institute (CLSI) 2022 guidelines, the combined disk method was employed for the screening of ESBL production among K. pneumoniae. In summary, the susceptibility of the organism 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 surrounding the ceftazidime/clavulanate and cefotaxime/clavulanate disks by a minimum of 5 millimeters compared to the disks lacking clavulanic acid (provided that the bacterial isolate is resistant to the agent when tested in isolation) (10). E. coli ATCC 35218 was utilized as the control strain in this study. A cefoxitin disk (30 µg) was utilized for the screening of AmpC-producing isolates. A double-disk synergy test was performed with cefoxitin-bronic acid to determine AmpC production (13). The Modified Hodge Test (MHT) was employed as a screening method for carbapenemase-producing isolates. K. pneumoniae ATCC BAA-1705 and BAA-1706 were utilized as MHT-positive and negative controls, respectively (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 previously described (15). In summary, the process of biofilm formation was initiated by cultivating bacterial isolates within a 96-well cell culture plate. The bacterial suspension was meticulously calibrated to achieve a turbidity of 0.5 McFarland, and 200 µL of the suspension was meticulously inoculated into each well. The inoculated wells were then subjected to an incubation process at a temperature of 37°C for a duration of 48 hours. Subsequently, the plates were subjected to three washes with Phosphate Buffered Saline (PBS), and each well was stained with 200 µL of 1% crystal violet for a period of 20 minutes at ambient temperature. Subsequent to the initial washing, the plates were subjected to a thorough cleansing process comprising three additional washings. This procedure was implemented to ensure the complete removal of extraneous stains. The crystal violet that had been attached to the adherent bacteria was solubilized with 180 µl of 33%

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. pneumonia.

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*

glacial acetic acid, and the resulting turbidity was measured at OD570. Un-inoculated LB medium was utilized as a negative control, while the reference strain ATCC 700603 was selected as a positive control. The classification of biofilm formation into four distinct groups was determined using the following formulas: The presence of a biofilm was determined by measuring the optical density (OD) of the samples. If the OD was less than the ODc, the biofilm was not formed (negative). If the OD was between the ODc and 2xODc, the biofilm was weak. If the OD was between 2xODc and 4xODc, the biofilm was moderate. The strength of the biofilm was determined by measuring the ratio of 4xODc to OD. If the ratio was less than one, the biofilm was considered strong.

2.8. Detection of virulence Genes

In this study, HvKp was defined as follows: positive capsular types K1 and K2, positive siderophore genes ≥2 (entB, iutA, iucA, Irp2), or ≥2 positive capsule-regulating genes (magA, wcaG, rmpA), and positive adhesions (mrkA, mrkD, fimH). Non-hvKp is designated as CKp (classic K. pneumoniae) (16). The K. pneumoniae isolates were then subjected to a polymerase chain reaction (PCR) screen for the following virulence genes: The following genes were identified: type 1 fimbrial adhesin (fimH), type 3 fimbrial adhesin (mrkD), enterobactin (entB), aerobactin siderophore biosynthesis (iucA) and its captor (iutA), Yersiniabactin high-pathogenicity island (irp-2), capsular polysaccharide (magA, wcaG), hypercapsule. The following elements are of particular relevance in this study: the Regulator of mucoid phenotype (RMPA) and the type 3 fimbriae (MrkA). The primers utilized for the identification of these genes were designed employing Allele ID 6 software and BLAST, utilizing the program available on the NCBI website. The complete list of primer sequences utilized can be found in Table 3.

2.9. Statistical Analysis

Descriptive statistics were employed to assess the characteristics of the study. The Pearson chi-square test was employed to ascertain significant differences between proportions. P values of less than 0.05 were considered to be statistically significant. The statistical analysis was conducted using SPSS version 16.0 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Antimicrobial Susceptibility

Phenotypic detection tests and molecular methods were used to identify the presence of ESBL/carbapenemase-producing K. pneumoniae strains in 27/91 (29.6%) of K. pneumoniae isolates from hospitalized patients in ICUs. These strains were found to harbor at least one of the carbapenemase/ESBL-related genes. In ninety-one K. pneumoniae specimens, ESBL-associated genes were detected, including 19.7% blaTEM, 29.6% blaSHV, and 19.7% blaCTX-M. Furthermore, the presence of carbapenemase-related genes was identified in 17.5% of the isolates, with blaOXA-48-like genes accounting for 15.4% and blaNDM1 genes responsible for 2.1% of the cases. Among the 27 beta-lactamase-producing K. pneumoniae isolates, the presence of ESBL-associated genes (18 [66.7%] blaTEM, 27 [100%] blaSHV, and 18 [66.7%] blaCTX-M) and carbapenemase-related genes (16 [59.3%]) was detected. The prevalence rates of these genes were blaOXA-48-like 14 (51.9%), and blaNDM1 2 (7.4%), in carbapenem-resistant K. pneumoniae (CRKP). Conversely, the genes blaIMP, blaVIM, and blaKPC were not detected in any of the isolates. Additionally, the AmpC-associated genes were not detected in any of the strains. According to the results of the CLSI breakpoint and susceptibility testing, 16 out of 27 (59.3%) ESBL/carbapenemase-producing K. pneumoniae strains were categorized as MDR, while 11 out of 27 (40.7%) were categorized as XDR (see Table 4). The minimum inhibitory concentrations (MICs) of ESBL/CRKP isolates against tigecycline and colistin ranged from 0.25 to 0.5 mg/L and from 2 to 16 mg/L, respectively. Tigecycline demonstrated sensitivity against all ESBL/CRKP isolates. The study revealed that azithromycin demonstrated the highest resistance rate (100%), followed by ceftazidime (85.18%), and cefotaxime (92.5%) (Figure 1). The broth microdilution test was utilized to assess the susceptibility of the isolates to tigecycline and colistin, revealing 100% and 85.2% sensitivity, respectively (Table 4). Another antibiotic that demonstrated 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. In the present study, the presence of mcr-1-5 genes was not detected in K. pneumoniae isolates.

3.2. Molecular Typing

A thorough examination of four colistin-resistant K. pneumoniae specimens via MLST analysis yielded a variety of STs, which are enumerated below: The following items are present: ST3500, ST273, and two cases of ST2558.

3.3. Assessment of Biofilm Formation Capacity

A total of 27 K. pneumoniae isolates were selected for analysis, and the results indicated that all of them exhibited the capacity to form biofilms. Of these, 12 (44.44%) were found to have 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. pneumoniae isolates. All K. pneumoniae isolates were found to carry at least one biofilm-related gene. The 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 (Figure 2). However, the iucA gene was not detected in any of the isolates examined. The number of positive virulence genes determinants ranged from three to eight genes among any isolate. The identification of fimbriae genes revealed that the fimH gene was detected in 81.48% of isolates, while the mrkA gene was positive in only 22.22% of isolates.

3.5. The Correlation between Biofilm Formation and Antibiotic Resistance Phenotypes

The majority of strong biofilm-forming K. pneumoniae isolates were XDR. A comparison of the two groups revealed that while only 25% of MDR isolates were strong biofilm producers, 73% of XDR isolates were strong biofilm producers (Figures 3 and 4). It is noteworthy that the majority of XDR isolates were found to carry both magA and mrkA virulence genes. The majority of the XDR

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

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*

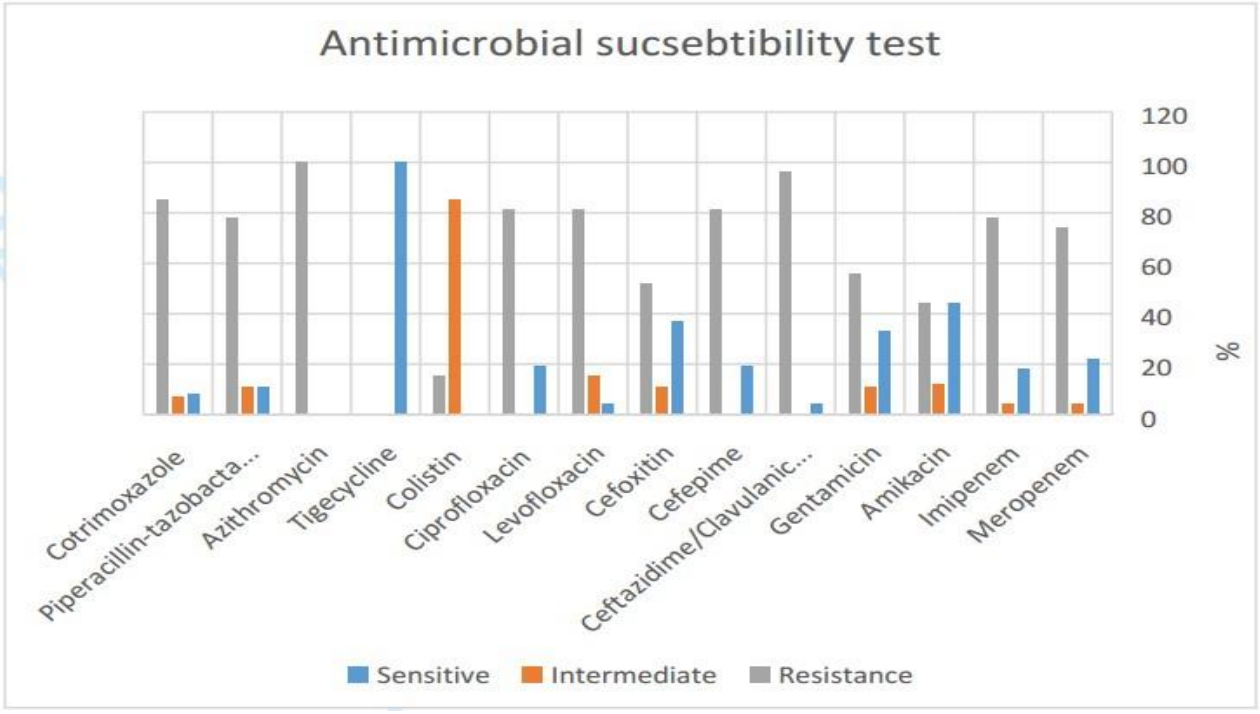

Figure 1. Diagram of the results of antibiotics susceptibility test.

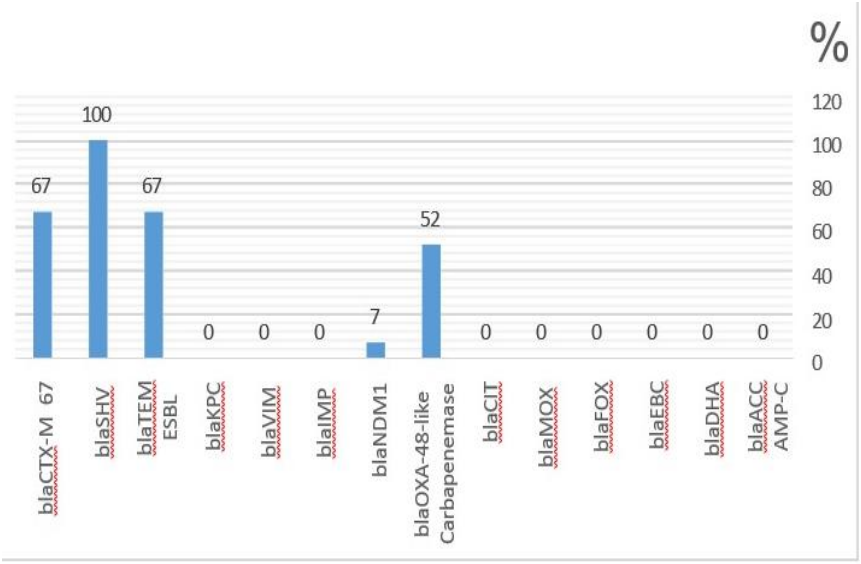

Figure 2. Diagram of the results of ESBL, AmpC, and Carbapenemase related genes.

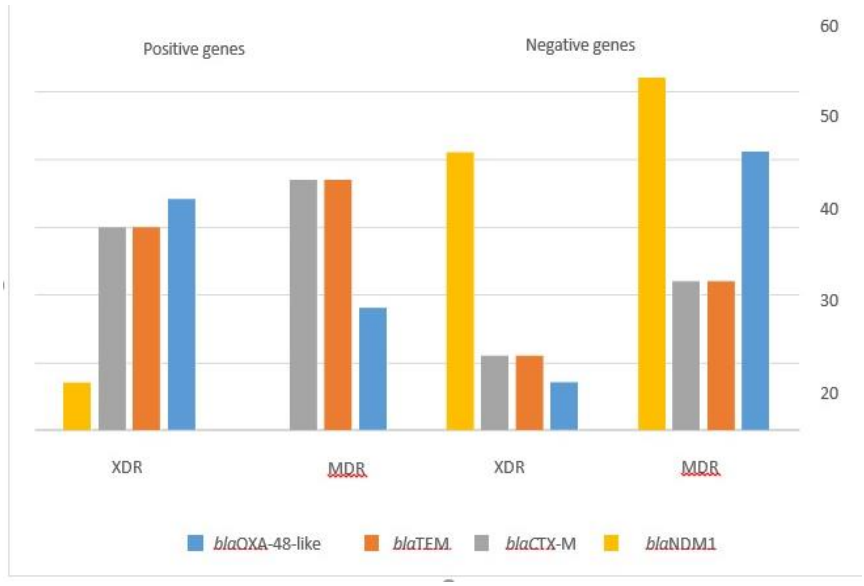

Figure 3. Comparative diagram of the results of antibiotics susceptibility test (MDR/XDR) and genes distribution.

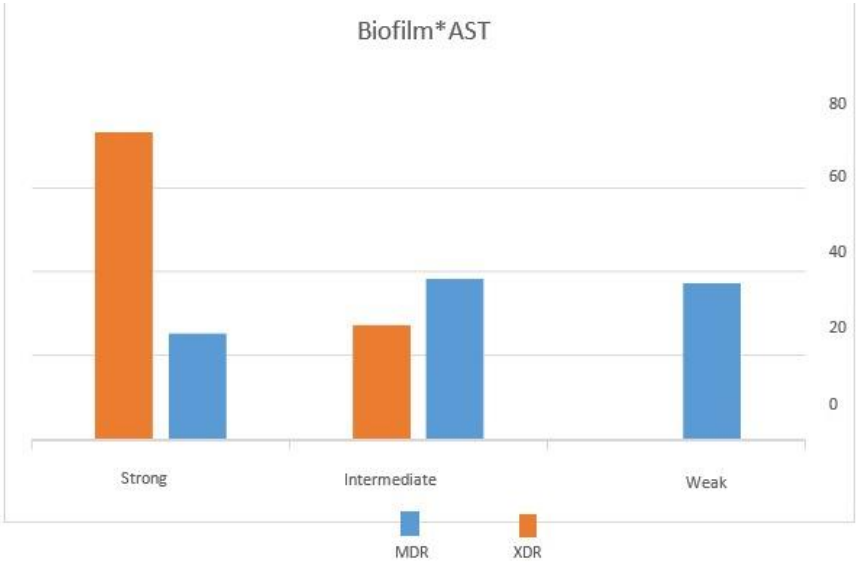

Figure 4. Diagram of biofilm production and AST and distribution of *fimH* and *magA* genes.

isolates were from the more virulent serotype of K1. In K1 isolates, the magA gene is imperative for the formation of the exopolysaccharide, a process that can be augmented by rmpA. In the present study, only one isolate was detected as hvkp (Table 4).

3.6. Association between the Presence of Virulence Genes and Biofilm Formation

According to the results of a polymerase chain reaction (PCR) assay designed for the detection of virulence genes, the fimH gene was not detected among five weak biofilm producers with K Non-Type. Furthermore, nine of the strong biofilm producers exhibited the magA gene, while one of the intermediate biofilm producers was found to be positive for this gene. The entB and mrkD virulence genes were positive in the majority of isolates. The irp2 gene's presence was confirmed among strong biofilm producers and three of the five moderate biofilm producers.

4. Discussion

The objective of the present 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, approximately one-third were found to be producers of ESBL and Carbapenem-resistant K. pneumoniae (CRKP). In this study, 50% and 56.2% of ESBL/CRKP isolates demonstrated resistance to meropenem and imipenem, respectively. In accordance with the findings of preceding studies (17), tigecycline emerged as the most efficacious antimicrobial agent against the isolates in question. In the present study, other antibiotics were observed to demonstrate higher sensitivities, with colistin exhibiting 85.2% sensitivity and amikacin exhibiting 44.4% sensitivity. The findings of this study are consistent with those of previous investigations, which examined the sensitivity of tigecycline (88.6% susceptibility) and colistin (73.9%) against carbapenem-resistant Enterobacterales (CRE) (18). According to recent reports, tigecycline has been identified as one of the most active antimicrobial agents against gram-negative and gram-positive isolates, including drug-resistant pathogens (19). Tigecycline remains the optimal treatment for MDR-CRE strains, attributable to its high degree of efficacy against these bacteria (19). Among the 27 K. pneumoniae isolates, 14 (51.8%) were found to be positive for the blaOXA-48–type gene. These isolates did not demonstrate the co-existence of other carbapenemases, with the exception of blaNDM-1. This finding is indicative of a high prevalence of OXA-48-positive K. pneumoniae in the present study. NDM-1 was the second most prevalent carbapenemase, identified in 7.4% of the isolates. Concurrently, the blaOXA-48 gene has been reported in the Middle East, and it is regarded as the most prevalent carbapenemase in Middle Eastern countries (20). The blaNDM1 gene was initially identified in India and has since been documented in Europe, North America, Asia, and Australia (20). The concomitant presence of blaNDM and blaOXA-48 genes in K. pneumoniae has been documented in multiple nations. The high prevalence of blaOXA-48 and blaNDM1 genotypes may be explained by the fact that Iran takes a large number of immigrants or visitors from countries with high prevalence of blaOXA-48 and blaNDM. Furthermore, the study indicated that three distinct types of enzymes (VIM, IPM, and KPC) were not of significant importance as carbapenemases. The results obtained in this study are consistent with the findings reported by Gheitani et al. The aforementioned researchers found that the prevalence rates of blaVIM, blaIMP, and blaKPC were 4 (2.18%), 1 (0.5%), and 0%, respectively (21). The findings of the present study demonstrated a considerable prevalence of SHV, CTX-M, and TEM enzymes among ESBL-producing K. pneumoniae strains in intensive care unit (ICU) patients with acute respiratory distress syndrome (ARDS) due to severe acute respiratory syndrome (SARS)-CoV-2. The findings of this study are consistent with those of other research conducted in Iran and other regions worldwide (22). The results of this study indicate that blaSHV is the predominant genotype among isolates. The situation related to ESBL production in Iran is very different, ranging from 9.8% to 75.7% (23). In the present study, no genes related to blaAmpC were detected. In contrast, the results of the present study may be inconsistent with those of surveys conducted in other regions of the world, possibly due to genetic variations in causative strains, antibiotic usage, and access to various antibiotic classes, including new ones (24). In the present study, the MDR/XDR isolates were found to harbor ESBL/CRKP genes, thereby rendering most antibiotic monotherapies ineffective. A further investigation, carried out in 18 European countries, has indicated that the rate of tigecycline resistance to carbapenem-resistant Enterobacterales is 88.6%, a finding that is consistent with the results of our own study (25). Colistin and certain aminoglycosides have demonstrated in vitro activity against carbapenem-resistant Enterobacterales. It has been posited that the combination of the pharmacodynamics of colistin and tigecycline is more efficacious against MDR/XDR isolates harboring ESBL/CRKP genes and mcr genes (26). The combination of therapy with the prevention of increased resistance to colistin and the ability to decrease colistin and tigecycline minimum inhibitory concentrations (MICs) (27) is a novel approach to combatting antimicrobial resistance. The rise in antibiotic resistance among biofilm-producing isolates is a serious concern, as it limits treatment options in hospitals. According to the findings of the surveys, it is imperative that substantial actions and the introduction of new strategies be considered to effectively address K. pneumoniae biofilm-related infections. The present study revealed that the majority of XDR isolates exhibited a propensity to develop more robust biofilms in comparison to MDR isolates. This observation suggests a direct correlation 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

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

Type of pathogens; resistance characteristic

Isolates	ESBL genotype	Carbapenemase genotype	MIC (mg/L)			MDR/XDR	Biofilm	AST	Capsule serotyp	CPS biosynthesis			Adhesion			Siderophores				Patient characteristics
			Colistin	Tigecycline	Azitromycinn					*rmpA*	*magA*	*wcaG*	*mrkD*	*mrkA*	*fimH*	*entB*	*iutA*	*iucA*	*Irp2*	
1	SHV, CTX-M, TEM	OXA-48	0.5	0.5	≥64	MDR	Intermediate	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	Intermediate	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	Intermediate	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	Intermediate	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, SXT, CAZ, CP, CTX, FOX	K1		+		+		+	+			+	64-year-old male with diabetes
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.

frequency than in the KPC-negative group (28). Consequently, the presence of genes of entB, magA, Irp2, fimH, and mrkD, as identified in our survey, underscores the necessity of evaluating these virulence factors. It is important to acknowledge the potential for variations in study outcomes due to differences in the study population. The findings of this study indicated the predominance of infections caused by β-lactamase-producing K. pneumoniae, which are biofilm producers, in ICUs. In the present study, all isolates exhibited robust and moderate biofilm production. The results of the study indicated that strong and moderate biofilm formation isolates must address new categories of antibiotics. The effective antimicrobial activity of tigecycline against bacteria that produce these enzymes may facilitate a more expeditious and efficacious treatment of hospitalized patients. The monitoring and control of nosocomial infections should be considered as a means of reducing the spread of MDR/XDR bacteria. These include surveillance systems that monitor changes in drug resistance profiles and etiology, the establishment of experimental treatment guidelines based on these profiles, and the proper instruction of healthcare workers regarding sanitation. Subsequent studies should encompass more intricate microbial communities present within healthcare facilities. Additionally, the field of study employing new antibiotics warrants attention.

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Authors' Contribution

The material preparation, data collection, and analysis were performed by S.R., M.B., F.N., and M.KH. The initial manuscript draft was authored by S.R. and M.B., with all authors providing feedback on successive iterations. H.E. made a significant contribution to the final version of the manuscript and provided oversight during the research process. S.R., M.B., H.S.S., and F.N. were responsible for the preparation of figures and tables.

Ethics
The present study was conducted with the approval of the ethics committee of Qazvin Medical University (approval number IR.QUMS.REC.1400.166). Moreover, the committee sanctioned the utilization of human samples. It is hereby confirmed that written informed consent to participate was obtained from all subjects in the study. Permissions and/or licenses to access the clinical patient data utilized in our research were obtained from Qazvin University of Medical Sciences. Hospitals were responsible for providing the clinical samples. It is imperative to acknowledge that the handling of biological samples in the present study is undertaken by the authors. The methods employed for the handling of human samples were executed in accordance with the pertinent guidelines and regulations stipulated in the Declaration of Helsinki. The research protocol was approved by the Research Ethics Committee at Qazvin Medical University in Iran.

Conflict of Interest
The authors declare that they have no competing interests.

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Data Availability
The datasets utilized and/or examined during the present study are available upon reasonable request to the corresponding author.

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