Correlation Between Antimicrobial Resistance and Biofilm Formation Capability Among Klebsiella Pneumoniae Strains Isolated From Hospitalized Patients in Iran

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Research

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Abstract

Background

*Klebsiella pneumoniae* (*K. pneumoniae*) is a common cause of nosocomial infections. Antibiotic resistance and ability to form biofilm, as two key virulence factors of *K. pneumoniae*, involved in persistent of the infections. The purpose of this study is to investigate the correlation between antimicrobial resistance and biofilm formation capability among *K. pneumoniae* strains isolated from hospitalized patients in Iran.

Methods

Over a 10-month period, a total of 100 non-duplicate *K. pneumoniae* strains were collected. Antibiotic susceptibility test was determined by Kirby-Bauer disk diffusion method according to CLSI. Biofilm formation was assessed by tissue culture plate method. Finally, polymerase chain reaction was conducted to detect four families of carbapenemase: *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>NDM</sub>, *bla*<sub>OXA-48</sub>, biofilm formation associated genes; *treC, wza, luxS* and *K. pneumoniae* confirming gene; *rpoB*.

Results

Most of the isolates were resistant to co-trimoxazole (52%), cefotaxime (51%), cefepime (43%), and ceftriaxone (43%). Among all the 100 isolates, 67 were multidrug-resistant (MDR), and 11 were extensively drug-resistant (XDR). The prevalence of the *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub> genes were 7%, 11%, 5%, and 28%, respectively. Among these isolates, 25% formed fully established biofilms, 19% were categorized as moderately biofilm-producing, 31% formed weak biofilms, and 25% were non-biofilm-producers. Molecular distribution of biofilm formation genes revealed that 98%, 96%, and 34% of the isolates carried *luxS*, *treC*, and *wza* genes, respectively.

Conclusion

The rise of antibiotic resistance among biofilm-producer strains, demonstrating a serious alarm about limited treatment options in hospital setting. Also, fundamental actions and introduction of novel strategies for controlling of *K. pneumoniae* biofilm-related infections is essential.

Background

*Klebsiella pneumoniae* (*K. pneumoniae*) has been clinically identified as one of the most important opportunistic pathogens responsible for nosocomial infection or healthcare-associated infections including, septicemia, urinary tract infections, soft tissue infections, and pneumonia [1]. Today, the increasing rate of drug resistance among *K. pneumoniae* isolates is a main concern worldwide [2]. Multidrug-resistant *K. pneumoniae* (MDR-Kp), which is resistant to many commonly used antibiotics such as aminoglycosides, fluoroquinolones, cephalosporins, and carbapenems, has been increasingly reported from Iran (3). MDR-Kp is a subject of great concern as it not only causes severe and fatal disease, but also increases the length of hospitalization, resulting in increased treatment charges [4]. Carbapenems are a class of highly effective antibiotic agents versus infections caused by MDR-Kp strains, though its application in administration of infections is threatened by development of carbapenem-resistant *K. pneumoniae* (CR-Kp) strains [3, 5]. CR-Kp strains are produced in response to a combination of one or more of the following mechanisms: stable derepression of AmpC, efflux pump overexpression, low outer membrane permeability, altered penicillin-binding proteins (PBPs), or production of class B metallo-β-lactamases (MBLs) and carbapenem-hydrolysing class D oxacillinases [6, 7]. So, one of the most important causes of resistance to *K. pneumoniae* is the biofilm formation, bacteria surrounded in an extracellular polysaccharide (EPS) matrix, and antibiotic impermeability [8, 9]. Formation of a biofilm, protects the bacteria from being eliminated by phagocytic cells. In addition, the bacteria in biofilms are more tolerant of antibiotics than those in planktonic form. Meanwhile, the resulting resistance to antimicrobials has been shown to hamper therapy [10, 11]. Some virulence-related genes, including cps gene cluster (a capsule encoding gene), *mrk* gene (type III fimbriae), *wbbM*, and *wzm* (LPS-synthesis-related genes) are involved in the biofilm production [12]. In addition, LuxS (type II quorum-sensing regulatory system) and *pgaABCD* operon, which are responsible for synthesis of poly-β-1, 6-N-acetyl-D-
glucosamine (PGA) (PgaC and PgaD) and secretion of PgaA and PgaB adhesions, affect biofilm development by increasing cell-to-cell interactions as well as abiotic surface binding and intercellular adhesion [13]. Though, it seems that, antimicrobial resistance and bacterial tendency to biofilm production play a key role in the emergence of MDR-\textit{Kp} strains, but, the clear correlation between these traits has not been completely cleared and elaborated. Thus, the purpose of this study is to investigate the correlation between antimicrobial resistance and biofilm formation capability among \textit{K. pneumoniae} strains isolated from hospitalized patients.

**Methods**

**Sampling and bacterial isolation**

In a 10-month period from April 2016 to January 2017, this cross-sectional study was performed on patients referring to four educational teaching hospitals in Sari, North of Iran. Microbial isolates were initially identified using conventional tests including Gram staining, indole production, motility, lactose fermentation, hydrogen sulfide (H2S) production, citrate and urease test, lysine decarboxylase and MR-VP and subsequently, considered by API20E kit (bio-Merieux, France) [3]. Species identification was confirmed by \textit{rpoB} gene PCR. Each \textit{K. pneumoniae} isolate was preserved in Trypticase Soy Broth (TSB) (Merck Darmstadt, Germany) with 20% glycerol at -70 °C.

**Antimicrobial Susceptibility Testing**

According to the clinical and laboratory standards Institute protocol (CLSI; M100-S14) [14], the antibiotic susceptibility was tested by disk agar diffusion method on the Mueller-Hinton agar plates (MHA) (Merck, Darmstadt, Germany) for ceftazidime (CAZ: 30 µg), cefotaxime (CTX: 30 µg), imipenem (IPM: 10 µg), meropenem (MEM: 10 µg), ciprofloxacin (CIP: 5 µg), cefepime (FEP: 30 µg), ceftriaxone (CRO: 30 µg), amikacin (AN: 30 µg), gentamicin (GM; 10 µg), and Trimethoprim-sulfamethoxazole (SXT; 5 µg) (MAST Diagnostics, Merseyside, UK).

Strains non-susceptible to at least three or more antimicrobial classes were defined as MDR, but, those non-susceptible to at least one agent in all but two or fewer antimicrobial categories were considered as XDR and the strains that non-susceptibility to all agents in all antimicrobial categories were defined as pan drug-resistant (PDR) [3]. \textit{Escherichia coli} ATCC 25922 was used as control organism in susceptibility testing.

**Phenotypic detection of MBLs**

CR-\textit{Kp} strains were assessed for MBL production using the double-disk synergy test (DDST). Briefly, a 0.5 McFarland turbidity (\(1.5 \times 10^8\) CFU/mL) of the microbial suspension was plated on MHA. Then, two 10-µg IPM disks were located on the MHA plates. Following this, 10 µL of MBL inhibitor (iMBL; 0.5 M EDTA) was directly added to one of the disks to reach a desired concentration of 750 mg. After an overnight incubation period at 37 °C, inhibition zone diameter (IZD) of all disks was recorded and compared. The strains were recognized as an MBL-producing isolate when the difference in the IZD was \(\geq 7\) mm [15].

**Quantitative biofilm assay**

Tissue culture plate (TCP) assay was used for quantitative measurement of biofilm production in \textit{K. pneumoniae} isolates. For each isolate, several colonies were inoculated in 10 mL of TSB with 1% glucose (TSBG) and incubated for 24 h at 37 °C in stationary phase; after incubation each suspension (adjusted to 0.5 McFarland (\(1.5 \times 10^8\) CFU/mL) with normal saline (0.85% NaCl) was diluted 1:100 in fresh TSB. Each wells of sterile 96-well microtiter plates (Sigma-Aldrich, USA) were filled with 200 µl of microbial suspension. As the negative control, sterile TSBG was employed, while the \textit{K. pneumoniae} ATCC 13883 was used as the strong biofilm producer positive control. The wells were washed four times with 0.2 mL of phosphate buffer saline (PBS, pH 7.2), desiccated for 1 h at 60 °C and stained for 15 min with 180 µl of 2% Hucker's crystal violet (0.1% w/v). The dye bound to the adherent cells was solubilized with 180 µl of 33% (v/v) glacial acetic acid (Zorka Pharma, Sabac, Serbia) per well and the absorbance was measured at 570 nm. Each assay was performed in triplicate, and repeated four times. The OD cut-off (ODc) was declared as three standard deviations above the mean OD of the negative control. Biofilm formation was recorded as follows: non-biofilm forming (\(A_{570} < 1\)); +, weak (\(1 < A_{570} < 2\)); ++, moderate (\(2 < A_{570} < 3\)); ++++, strong (\(A_{570} > 3\)) [16].
Molecular examination

PCR was utilized for detection of MBL genes (bla<sub>IMP</sub>, bla<sub>VIM</sub>, bla<sub>NDM</sub> and bla<sub>OXA-48</sub>), biofilm-encoding genes (treC, wza and luxS) and <i>K. pneumoniae</i> confirming gene (rpoB). Bacterial DNA template was obtained from the purified colonies grown on the brain heart infusion agar plates (Merck, Darmstadt, Germany) using a bacterial genomic DNA extraction kit (Bioneer, Daejeon, Korea), and then kept at -20 °C. The oligonucleotide primer sequences used in the present work are shown in Table 1 [13, 16–18]. The PCR reaction was performed in a final volume of 25 µl, including the following ingredients: 1.0 µL of extracted DNA, 12 µL Maxima Hot Start PCR Master Mix (2×) (Thermo, Waltham, Massachusetts, United States), 0.8 µL of each primer, and 10.4 µL of sterile distilled water. The samples were amplified in thermal cycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 94 °C for 7 min, followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 60 s, extension at 72 °C for 60 s and a final extension at 72 °C for 5 min. PCR products were electrophoresed on 1.5% agarose gel stained with Gel Red™ (Biotium, Landing Pkwy, Fremont, CA, USA), then photographed under an ultraviolet transilluminator (Bio-Rad, Hercules, USA). Both positive and negative controls were used in PCR for optimization and standardizing of molecular test.

Table 1
List of primers used for PCR amplification

| Target gene | Primer sequences (5’→3’) | Product size (bp) |
|-------------|--------------------------|------------------|
| rpoB        | 5'- CAACGGTTGTGTTACTGACG-3\<sup>\</sup>  
5'- TCTACGAAGTGGCCGTTTTC-3\<sup>\</sup> | 108               |
| bla<sub>IMP</sub> | 5'- TGAGCAAGTTATCTGTATTC-3\<sup>\</sup>  
5'- TTAGTTGCTTGGTTTTGATG-3\<sup>\</sup> | 740               |
| bla<sub>VIM</sub> | 5'- GATGGTGTGTGTCCGACATA-3\<sup>\</sup>  
5'- CGAATGCGCAGCACCAG-3\<sup>\</sup> | 390               |
| bla<sub>NDM</sub> | 5'- GCTTGGCGATCTGGTTTTTC-3\<sup>\</sup>  
5'- CGGAATGGCTCATCAGATC-3\<sup>\</sup> | 621               |
| bla<sub>OXA-48</sub> | 5'- TTGGTGGCATCAGATGTCAGG-3\<sup>\</sup>  
5'- GAGCAGTTTTGATGGCGG-3\<sup>\</sup> | 428               |
| treC        | 5'- CGGACACCGGGCAGATT-3\<sup>\</sup>  
5'- CGCCCGATTCTCCAGTT-3\<sup>\</sup> | 71                |
| wza         | 5'- CGCAGTGATGCGTATT-3\<sup>\</sup>  
5'- TGACTGCGTTCTGATGC-3\<sup>\</sup> | 309               |
| luxS        | 5'- GCCGTTGTAGATATGTTCACAG-3'  
5'- CAGTTCCTGTTGCTGTGATG-3' | 447               |

Data analysis

SPSS software version 16 for windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Statistical significance in this study was <i>P</i> < 0.05.

Results

Bacterial isolation
In total, 100 non-duplicative clinically-relevant *K. pneumoniae* were collected from urine (n = 61), wound exudates (n = 13), intratracheal tube (ITT) (n = 11) blood (n = 9), and sputum (n = 6). The prevalence of isolates in hospital wards was as follow: Intensive Care Units (ICUs) (n = 31), burn (n = 24), dialysis (n = 16), hematology-oncology (n = 9), respiratory care (n = 7), internal medicine (n = 6), neonatal intensive care unit (NICU) (n = 5), and surgery (n = 2). It worth mentioning that the positive culture in urine specimens was defined as a growth of more than $10^5$ CFU/mL. The mean age of the patients was 49.6 years (range, 18 to 86 years), where 60% (n = 60) patients were female and 40% (n = 40) were male.

**Antimicrobial resistance profile**

Based on the acquired antibiotic resistance pattern, all *K. pneumoniae* strains were considered resistant to at least one of the tested antibiotics. In detail, 67% of the isolates resulted resistant to three or more antimicrobials, and 11% isolates were classified as XDR. No PDR isolate was identified. As presented in Table 2, most of the isolates were resistant to SXT (n = 52/100, 52%), CTX (n = 51/100, 51%), FEP (n = 43/100, 43%), and CRO (n = 43/100, 43%). Further, the lowest resistance rate was related to the AN (n = 7/100, 7%), all of which were MDR-

| Susceptibility profile | SXT | CTX | FEP | AN | IPM | MEM | CRO | GM | CAZ | CIP |
|-----------------------|-----|-----|-----|----|-----|-----|-----|----|-----|-----|
| MDR                   |     |     |     |    |     |     |     |    |     |     |
| S                     | 25  | 26  | 27  | 55 | 41  | 38  | 29  | 48 | 28  | 19  |
| (37.3%)               | (38.8%) | (40.3%) | (82.1%) | (61.2%) | (56.7%) | (43.3%) | (71.6%) | (41.7%) | (28.3%) |
| I                     | 8   | 5   | 5   | 2  | 1   | 9   | 2   | 8  | 4   |
| (5.9%)                | (11.9%) | (7.5%) | (7.5%) | (2.9%) | (1.5%) | (13.4%) | (2.9%) | (11.9%) | (5.9%) |
| R                     | 38  | 33  | 35  | 7  | 24  | 28  | 29  | 17 | 31  | 44  |
| (56.7%)               | (49.3%) | (52.2%) | (10.4%) | (35.8%) | (41.7%) | (43.3%) | (25.4%) | (46.3%) | (65.6%) |
| Non-MDR               |     |     |     |    |     |     |     |    |     |     |
| S                     | 18  | 22  | 22  | 31 | 22  | 23  | 16  | 25 | 18  | 19  |
| (54.5%)               | (66.6%) | (66.6%) | (93.9%) | (66.6%) | (69.6%) | (48.5%) | (75.5%) | (54.5%) | (57.5%) |
| I                     | 1   | 4   | 3   | 2  | 0   | 1   | 3   | 0  | 2   | 5   |
| (3%)                  | (12.1%) | (9.1%) | (6.1%) | (0.0%) | (3%) | (9.1%) | (0.0%) | (6.1%) | (15.2%) |
| R                     | 14  | 18  | 8   | 0  | 11  | 9   | 14  | 8  | 13  | 9   |
| (42.4%)               | (54.5%) | (24.2%) | (0.0%) | (33.3%) | (27.3%) | (42.4%) | (24.2%) | (39.4%) | (27.3%) |

Abbreviations: MDR; multidrug resistant, S; susceptible, I; intermediate resistant, R; resistant, SXT; Trimethoprim-sulfamethoxazole, CTX; cefotaxime, FEP; cefepime, AN; amikacin, IPM; imipenem, MEM; meropenem, CRO; ceftriaxone, GM; gentamicin, CAZ; ceftazidime, and CIP; ciprofloxacin.

**Biofilm production**

Our data revealed that 75% of *K. pneumoniae* isolates were biofilm-producing isolates. The isolates were divided into four categories as defined above. In this study, 31%, 25%, and 19% of isolates were weakly, strongly, and moderately biofilm-producing strains, respectively. In addition, 25% of the isolates were considered non-biofilm producers. The prevalence of biofilm formation in MDR isolates was significantly higher than in non-MDR isolates (p < 0.05) (51% compared to 24%) (Table 3). The biofilm-formation ability among the isolates collected from sputum was significantly higher compared to the other isolates (P < 0.001). Also, antimicrobial resistance pattern of *K. pneumoniae* among biofilm formers and non-formers along with the susceptibility pattern of the tested isolates was shown in Table 4. The antimicrobial resistance among biofilm producing *K. pneumoniae* strains was found significantly higher than that of biofilm non-producing *K. pneumoniae* strains (p < 0.05). The correlation between biofilm production and antimicrobial resistance was found statistically significant (p < 0.05) in most of the
antibiotics from different classes; CAZ, IPM, MEM: CIP, FEP, CRO, AN, and GM, but the correlation was not found to be significant in case of SXT and CTX.

Table 3
Biomf formation in MDR and non-MDR *K. pneumoniae* isolates

| Clinical Isolates | Biomf Model
|-------------------|------------------|------------------|------------------|------------------|
|                   | None (0)         | Weak (+)         | Moderate (+++)   | strong (+++)     |
| MDR               | 8 (11.9%)        | 23 (34.2%)       | 8 (11.9%)        | 20 (29.8%)       |
| Non-MDR           | 17 (51.5%)       | 8 (24.2%)        | 11 (33.3%)       | 5 (15.2%)        |

Table 4
Antimicrobial resistance pattern of *K. pneumoniae* among biomf producer and non-producer strains

| Biofilm formation ability | Strong (n = 25) | Moderate (n = 19) | Weak (n = 31) | Non-biomf formation (n = 25) |
|--------------------------|-----------------|-------------------|---------------|-----------------------------|
| Antibiotics              | R No. (%)       | I No. (%)         | S No. (%)     | R No. (%)       | I No. (%)     | S No. (%)     | R No. (%)       | I No. (%)       | S No. (%)     |
| SXT                      | 13 (52)         | -                 | 11 (57.8)     | 7 (36.8)        | 12 (38.7)     | 16 (51.6)     | 16 (68)         | 1 (4)           | 8 (28)         |
| CTX                      | 15 (60)         | -                 | 12 (63.1)     | 5 (26.3)        | 14 (45.1)     | 13 (41.9)     | 9 (36)          | 4 (16)          | 12 (48)        |
| FEP                      | 12 (48)         | 3 (12)            | 10 (52.6)     | 8 (42.1)        | 11 (35.4)     | 19 (61.2)     | 9 (36)          | 3 (12)          | 13 (52)        |
| AN                       | 4 (16)          | 1 (4)             | 20 (80)       | 16 (84.2)       | 27 (87.09)    | 1 (4)         | 1 (4)           | 23 (92)         |
| IPM                      | 13 (52)         | 1 (4)             | 11 (44)       | 9 (47.3)        | 10 (52.6)     | 24 (77.4)     | 7 (28)          | -               | 18 (72)        |
| MEM                      | 14 (56)         | -                 | 11 (44)       | 7 (36.8)        | 11 (57.8)     | 23 (74.1)     | 8 (32)          | 1 (4)           | 16 (64)        |
| CRO                      | 13 (52)         | 2 (8)             | 10 (40)       | 11 (57.8)       | 7 (36.8)      | 10 (32.2)     | 8 (32)          | 4 (16)          | 13 (52)        |
| GM                       | 8 (32)          | -                 | 17 (68)       | 4 (21.05)       | 13 (68.4)     | 22 (70.9)     | 3 (12)          | 2 (8)           | 20 (80)        |
| CAZ                      | 12 (48)         | 4 (16)            | 9 (36)        | 12 (63.1)       | 1 (5.2)       | 13 (41.9)     | 10 (40)         | 2 (8)           | 13 (52)        |
| CIP                      | 17 (68)         | 3 (12)            | 5 (20)        | 12 (63.1)       | 7 (36.8)      | 14 (45.1)     | 13 (41.9)       | -               | 13 (52)        |

Abbreviations: R; resistant, S; susceptible, I; intermediate resistant, SXT; Trimethoprim-sulfamethoxazole, CTX; cefotaxime, FEP; cefepime, AN; amikacin, IPM; imipenem, MEM; meropenem, CRO; ceftiraxone, GM; gentamicin, CAZ; ceftazidime, and CIP; ciprofloxacin.

The distribution of studied genes

Based on the PCR results, the MBLs genes identified in this study were *bla*<sub>VIM</sub> (n = 7, 7%), *bla*<sub>IMP</sub> (n = 11; 11%), *bla*<sub>NDM</sub> (n = 5; 5%), and *bla*<sub>OXA-48</sub> (n = 28, 28%). The molecular distribution of biofilm formation genes among the isolates indicated that, the most
prevalent biofilm encoded genes were luxS with (98%), treC with (96%) and wza with (34%). Finally, the co-presence of biofilm encoded factors was as follows: luxS/ treC/wza (n = 21), luxS/treC (n = 62), luxS/wza (n = 14), and treC/wza (n = 8).

**Discussion**

The increasing number of resistant *K. pneumoniae* strains to multiple antibiotics is a major challenge in medical centers worldwide. Heidary et al. (2018), in a systematic review and meta-analysis article, showed that there is a relatively high prevalence of drug resistant *K. pneumoniae* isolates in Iran [19]. The highest resistance rate in the *K. pneumoniae* isolates was observed against ampicillin (82.2%), aztreonam (55.4%), and nitrofurantoin (54.5%), while, in the present study, 52%, 51%, 43%, and 43% of isolates were resistant to SXT, CTX, FEP, and CRO, respectively. Khamesipour et al. (2016) indicated widespread resistance to CRO (41.1%), SXT (36.7%), AN (32.2%), FEP (34.4%) and GM (26.7%) [20]. As well as, in the study of Moghadas et al. (2018), the antibiotic resistance rates were as follows: IPM (7.5%), CIP (16.1%), SXT (32.9%), FEP (34.1%), AN (36.4%), and CAZ (42.7%) [21]. In contrast to our study, 89.5% of isolates were MDR in Hou et al. (2015) study [22]. This percentage was far higher than those reported in our study. This discrepancy may be related to geographic distance, antimicrobial-prescribing patterns in hospitals and level of hygiene. According to the results, in DDST, of 35 IPM-resistant *K. pneumoniae* isolates, 74.3% were MBL-positive. The prevalence of *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>NDM</sub>, and *bla*<sub>OXA-48</sub> was 7%, 11%, 5%, and 28%, respectively. In contrast to the present study, Carroll et al. (2013) reported that all isolates were negative for *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, and *bla*<sub>SPM</sub> genes [23]. An interesting point in this study was the presence of *bla*<sub>NDM</sub> gene. The presence of *bla*<sub>NDM</sub> gene in *K. pneumoniae* was first reported by Shahcheraghi et al. (2010) [24]. According to Fallah et al. (2014), *bla*<sub>NDM</sub> is an MBL-encoding gene, which was newly recognized and described from New Delhi, India, for the first time followed by other areas such as Pakistan. Neighborhood of these countries with our country and many travels between the countries on the one hand and the ease of resistance transfer among microorganisms on the other led us to postulate that our isolates are likely have the same gene [25]. In the study of Sei et al. (2016), of 94 *K. pneumoniae* isolates, 33% formed fully established biofilms, 52.1% were categorized as moderately biofilm-producing, 8.5% formed weak biofilms, and 6.4% were non-biofilm-producers [26]. Li et al. (2012) suggested that the expression of different adhesion, their cognate receptors, and exopolymeric components by individual cell types within a biofilm community can contribute to the general biofilm development. In particular, many bacteria are capable of using a quorum sensing mechanism to regulate biofilm formation and other social activities [27]. In this study, most of the biofilm producer strains were MDR. Our data revealed that 75% of *K. pneumoniae* were biofilm-producing isolates. These data are similar with the findings of Sei et al, (2016) [26]. Zheng et al. (2018) found that biofilm formation was more pronounced among *magA* (K1), *aero+*, *mpaA+*, *mpaA2+*, *allS+*, *wcaG+*, and *iutA+* isolates than in isolates which were negative for these virulence factors [18]. Wu et al. (2011) concluded that *treC* and *sugE* affect biofilm formation by modulating capsular polysaccharide (CPS) production [13]. The importance of *treC* in gastrointestinal tract colonization suggests that biofilm formation contributes to the establishment and persistence of *K. pneumoniae* infection. In agreement with Sei et al. (2015) and Boisvert et al. (2016), strong-biofilm producing phenotypes were higher in strains isolated from sputum samples compared to other specimens [26, 28]. This indicates the important role of biofilms in the survival and colonization of microbes in the lungs, causing bacterial resistance to pulmonary clearance. In addition, previous study showed that luxS was shown to be upregulated in biofilm-grown XDR *K. pneumoniae* strains [12]. Notably, in our study the luxS gene was detected in about 98% of the tested isolates. Using a rat model of middle ear challenge, Yadav et al. (2018) demonstrated that the functional defect in LuxS, leading to the reduced colonization capability of pneumococci in vivo [29]. However, in contrast to our results, Pakhshan et al. [30] concluded that the susceptible isolates to antibiotics tend to form stronger biofilms compared with the resistant strains.

**Conclusions**

MDR-*Kp* is becoming a serious problem in hospitals, with many strains developing resistance to most available antimicrobials. The increasing rate of CR-*Kp* strains emphasizes the importance of choosing an appropriate antimicrobial regimen based on antibiotic susceptibility pattern. Also, the distribution of MBL producing strains is an important problem due to high antimicrobial resistance rate of them, requiring the routine evaluation and changing the antibiotic stewardship according to the results. Our findings supported the role of biofilm formation in resistance to antimicrobial agents. Further research on the
mechanisms of biofilm formation in *K. pneumoniae* will ultimately assist in the treatment of biofilm-mediated infections and in the reduction of mortality and morbidity in patients suffering from life-threatening nosocomial infections.

**Abbreviations**

MDR-Kp
Multidrug-resistant *K. pneumoniae*; CR-Kp: carbapenem-resistant *K. pneumoniae*; MBLs: metallo-β-lactamases; H2S: hydrogen sulfide; TSB: Trypticase Soy Broth; PCR: polymerase chain reaction; CLSI: Clinical Laboratory Standards Institute; MDR: multidrug-resistant; XDR: extensively drug-resistant; PDR: pan drug-resistant; DDST: double-disk synergy test; TSBG: TSB with 1% glucose; TCP: Tissue culture plate; ODc: Optical Density cut-off;

**Declarations**

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**Authors' contributions**

MA: Design of the study and supervision. SS: collected the data, cultured the samples and performed experiments. HG Advisor in the study and contributed to the analysis of the data in collaboration with BM. MG: Assisted in molecular examinations and edited the manuscript. MG drafting of the manuscript in collaboration with HG. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this work are included in this published article. Also, the all data used to support the findings of this study are available from the corresponding author upon request.

**Ethics approval and consent to participate**

The study was approved by the ethical committee of Mazandaran University of Medical Sciences, Sari, Iran (MAZUMS) (Consent Ref: NO.14.5.1396). The informed consent was obtained from all the participants or a close relative. Identifying information of each specimen was kept no-name and secret.

**Consent for publication**

There is no limit to the publication. All authors consented for the publication of this research.

**Conflict of interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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