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Original Article

Comparison of biofilm formation and efflux pumps in ESBL and carbapenemase producing *Klebsiella pneumoniae*

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Abstract

Introduction: *Klebsiella pneumoniae* is an opportunistic pathogen that causes a range of diseases. The appearance of extended-spectrum β-lactamase -and carbapenemase-producing strains, in addition to the biofilm-forming phenotype, is a major problem in the clinical environment.
Methodology: A total of 33 clinical *K. pneumoniae* isolates were used in this study. Antimicrobial susceptibilities were assessed by a disc diffusion assay. Biofilm formation was determined by a microtiter plate assay, staining with 1% crystal violet and measuring absorbance after destaining. Moreover, expression of *acrA, kdeA, ketM, kpnEF,* and *kexD* efflux associated genes was measured by qRT-PCR.
Results: Isolates displayed high resistance to β-lactams such as cefazolin, cefuroxime, ceftriaxone, cefepime, piperacillin-tazobactam, imipenem, and meropenem and decreased resistance to gentamicin, amikacin, ciprofloxacin, and levofloxacin. ESBL-producing isolates formed more biofilm than carbapenemase-producing isolates. The mRNA expression levels in KPC isolates for *acrA* (2-fold), *kdeA* (2.7-fold), *ketM* (2.2-fold), and *kpnEF* (3.4-fold) were significantly increased compared to ESBL-producing isolates. There was no significant difference in *kexD* expression level.
Conclusions: Under the conditions used here ESBL-producing isolates formed more biofilm than KPC postive isolates; this was associated with virulence determinants which were also transferred by plasmids together with ESBLs enzymes. Moreover, the upregulation of *acrA, kdeA, ketM,* and *kpnEF* efflux pumps was seen in carbapenemase-producing isolates demonstrating that high expression of efflux pumps alone could not confer resistance but may act as a physiological determinant such as bacterial pathogenicity and virulence, and cell-to-cell communication for bacteria.

Key words: *Klebsiella pneumoniae*; antibiotic resistance; biofilm formation; efflux pumps.

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Introduction

Klebsiella pneumoniae, a member of Enterobacteriaceae family, is a gram-negative opportunistic bacteria which can cause a wide range of nosocomial infections such as pneumonia, bacteremia, respiratory, and urinary tract infections (UTI) in immunocompromised patients [1]. Moreover, *Klebsiella pneumoniae* is one of the most clinically important organisms and frequently isolated strain from intensive care unit (ICU) patients and is responsible for approximately %15 of the gram-negative bacteria-related infections [1,2]. Besides the high prevalence, many strains are highly resistant to commonly used antibiotics and the remarkable increase has been seen in mortality and morbidity over the years [3].

Increasing antibiotic resistance has been observed in *K. pneumoniae*, including ESBL production. In addition, more recently, *Klebsiella pneumoniae* carbapenemase-producing (KPC) strains are one major concern around the world [4,5]. The rate of ESBL-producing bacteria was highest for *K. pneumoniae* isolates from Latin America (44%) followed by Asia pacific (22.4%), Europe (13.3%), and North America (7.5%) [6]. At the same time, the massive use of carbapenems has encouraged the progress of plasmid-mediated carbapenemase enzymes that hydrolyze all β-lactams including the "last-resort" carbapenems in the USA and subsequently worldwide [7,8]. With the increasing use of β-lactam antibiotics to treat ESBL/carbapenemase-producing bacterial infections, different resistance mechanisms become prominent and these mechanisms make bacteria more "shielded" against antimicrobial agents [9].

Bacterial efflux pumps are important membrane proteins, causing antibiotic resistance in many bacterial pathogens. These pumps provide resistance by ejecting

many antibiotics from cell and decrease the antibiotic concentration in the cell. Today, there are five families of bacterial efflux systems described: the major facilitator superfamily (MFS); ATP-binding cassette (ABC) family; resistance-nodulation-division (RND) family; multidrug and toxic compound extrusion (MATE) family; and small multidrug resistance (SMR) family. In addition to antimicrobial extrusion, efflux pumps can extrude a wide range of different compounds such as host-derived antimicrobials, solvents, dyes, detergents, bile, hormones and bacterial metabolites [10,11]. Although high expression of some efflux pumps makes bacteria more resistant to antibiotics, efflux pumps are commonly under the control of regulators which keep them in "stand by" mode to ensure energy consumption [12]. Many studies have demonstrated the role of efflux pumps in antibiotic resistance but the crosstalk between plasmid-mediated resistance and efflux pumps has not been fully understood yet.

Biofilms are defined as communities of microorganisms that are attached to a biotic or abiotic surface and live in a matrix of hydrated extracellular polymeric substances (EPS), Biofilms are often associated with resistance to antibiotics. As a sessile (attached) form, bacteria are phenotypically and genotypically different from their planktonic (free swiming) counterparts. Sessile cells reduce the penetration of antibiotics through the biofilm, show induced adaptive stress responses, increased efflux, and are more likely to be 'persister' cells [13]. Biofilm producing strains of *K. pneumoniae* have an important negative effect on human health and are associated with a wide range of diseases such as UTIs and ventilator-associated pneumonia (VAP) [14]. In recent years, common infections caused by ESBL- and carbapenemase-producing isolates have been related to increased biofilm formation [15].

The aim of this study was to evaluate antimicrobial susceptibility, expression levels of efflux pumps and biofilm-forming abilities among different resistance profiles of *K. pneumoniae* isolates from ICU patients.

Methodology

Bacterial strains, culturing and growth conditions

From January 2013 to January 2017, a collection of 33 *K. pneumoniae* were isolated from ICU patients in Amasya University Sabuncuoğlu Şerefeddin Education and Research Hospital, Turkey. Each isolate was collected from a different patient. ESBL- and carbapenemase-producing isolates were obtained from sputum samples. Sputum samples were inoculated into eosin methylene blue (EMB) agar (RTA, Kocaeli, Turkey), %5 sheep blood agar (RTA, Turkey), and chocolate agar (RTA, Turkey). Bacterial identification, ESBL and carbapenemase production were performed using VITEK 2 (bioMérieux, North Carolina, USA) automated system. All strains were routinely cultured in tryptic soy broth (TSB) (RTA, Kocaeli, Turkey) or tryptic soy agar (TSA) (RTA, Turkey) unless otherwise stated.

Phenotypic detection of ESBL production

The phenotypic confirmation of the ESBL-producing isolates was performed by using combined disc test with ceftazidime/clavulanic acid (CAZ/CLA) (30/10 µg) and ceftriaxone /clavulanic acid (CRO/CLA) (30/10 µg) combination discs. A $\geq$ 5 mm zone diameter increased around the cephalosporin disc combined with clavulanic acid compared the zone around the disc with the cephalosporin alone was confirmed as an ESBL positive isolate [16]. All antibiotics were purchased from MastDisk, UK. *K. pneumoniae* ATCC 700603 were used as reference strain.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion method, following Clinical & Laboratory Standards Institute (CLSI) recommendations [16]. The antimicrobials were tested with cefazolin (30 µg), cefuroxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), piperacillin/tazobactam (100/10 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), and levofloxacin (5 µg). All antibiotics were purchased from MastDisk, UK. Isolates with intermediate phenotype were considered as resistant. *Escherichia coli* ATCC 25922 were used as reference strain.

Biofilm formation

Biofilm formation was determined by the microtiter plate assay, as previously described [17]. Briefly, a 1:100 dilution was prepared from overnight grown isolates and 200 µL of this dilution were inoculated into 96-well flat bottom polystyrene plates (Greiner Bio-One Inc., Frickenhausen, Germany). The plates were then incubated at 37°C for overnight. Wells were washed twice with 200 µL of phosphate buffered saline (PBS) and dried at room temperature. The wells were stained with 0.1% crystal violet solution for 20 minutes and the plates were washed with distilled water and dried at room temperature. After the addition of 200 µl

of 95% ethanol, the optical density (OD) of the biofilms was measured at 595 nm using an ELISA reader (Multiscan GO, Thermo Scientific, Vantaa, Finland). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 15692 were used as negative and positive biofilm producers, respectively.

Total RNA Isolation and cDNA Synthesis

Overnight cultures were grown in TSB and allow to reach mid-logarithmic phase (OD_{600} of 0.7-1) at 37°C. Total RNA was extracted using GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The concentration and purity of total RNA samples were determined via spectrophotometer. The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania) from 100 ng of total RNA with Oligo $(dT)_{18}$ primers following the manufacturer's instructions. cDNA was stored at -20°C until required.

Real-time PCR

The relative expression of *AcrA, kexD, kdeA, kpnEF,* and *ketM* efflux pumps' genes was performed in PikoReal™ Real-Time PCR System (Thermo Scientific, Vantaa, Finland) using Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific, Vilnius, Lithuania) kit according to the manufacturer's instructions. The primers used for qRT-PCR were listed in Table 1. Briefly, the reaction mix was composed of 5 µL of Maxima SYBR Green qPCR Master Mix (2X), 0.5 µL of forward primer (0.5 µM), 0.5 µL of reverse primer (0.5 µM), 1 µL of cDNA, and 3 µL of nuclease free water in total 10 µL reaction volume. Melting curve analyses were performed to ensure specificity. Fold change of each gene was calculated using ΔΔCT method [18]. The fold change in gene expression was determined by normalization of each gene to the 16S rRNA internal control and relative to *K. pneumoniae* ATCC 700721 as a calibrator. Negative Template Control (NTC) was also used to detect contamination.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (Graphpad Software Inc, USA). Differences between groups were assessed by the Mann Whitney test. The data was expressed as the mean ± standard deviation (SD). p values of less than 0.05, 0.01 and 0.001 were selected as the level of significance.

Results

A collection of 33 *K. pneumoniae* were collected from ICU patients in Amasya University Sabuncuoğlu Şerefeddin Education and Research Hospital, Turkey. All *K. pneumoniae* isolates were obtained from sputum. These isolates were characterized according to their antibiotic susceptibility profile, biofilm formation, and expression of efflux pumps.

Susceptibilities of K. pneumoniae

According to the biochemical identification by VITEK 2 automated system, 17/33 of these isolates were ESBL producers while 16/33 of them were carbapenemase producers. The susceptibilities of *K. pneumoniae* isolates to different antimicrobial agents such as β-lactams and non-β-lactams were determined by disc diffusion method. Based on CLSI recommendations, high resistance rates were observed in β-lactams such as cefazolin 100% (33/33), cefuroxime 100% (33/33), ceftriaxone 100% (33/33), cefepime 100% (33/33), piperacillin-tazobactam 45% (15/33), imipenem 48% (16/33), and meropenem 48% (16/33). Among non-β-lactam antibiotics, significant decrease was observed in gentamicin 30% (10/33), amikacin 9% (3/33), ciprofloxacin 36% (12/33), and levofloxacin 27% (9/33). There was a decrease

Table 1. Primers used in this study.

Primer	Primer Sequence	Product Size	Annealing Temperature	Reference
kdeA-F	GTTGTTCCCGTTATGTCTGGTGC	170 bp	53°C	[40]
kdeA-R	CCAGCAGCCACTGTAAAAACATGC			
kexD-F	ACCGGTTGCGCAATACCCTGA	184 bp	53°C	[41]
kexD-R	CGTAATTGACGCCATCCCTG			
kpnE-F	ATTGCTGAAATTACCGGCAC	172 bp	51°C	[42]
kpnE-R	AAATACCGATCCCTTCCCAC			
ketM-F	TTGGCAGAGAAGGCGGTTGG	123 bp	51°C	[43]
ketM-R	CATGACCATCCCGGGCTTG			
16S-F	AGGCCTAACACATGCAAGTC	326 bp	52°C	[40]
16S-R	TGCAATATTCCCCACTGCTG			
AcrA-F	GTCCTCAGGTCAGTGGCATTA	257 bp	50°C	[44]
AcrA-R	ATTGCTCTGCTGCGCCGTT			

in resistance seen with the non β-lactams relative to the β-lactams. Antibiotic susceptibility profiles of the isolates were given in Table 2.

Biofilm formation of K. pneumoniae

Biofilm formation of all isolates was evaluated using microtiter plate assay. Biofilm production was classified according to biofilm-negative control (*E. coli* ATCC 25922). The optical densities (OD) of isolates which were higher than biofilm-negative control were counted as biofilm producers. Our findings indicated that 100% (33/33) of *K. pneumoniae* isolates were biofilm producers. To assess whether there was any difference between ESBL- and carbapenemase-producing isolates, two groups were compared. ESBL-producing isolates (n = 17) with the mean OD value of 0.46 formed more biofilm than carbapenemase-producing isolates (n = 16) with the OD value of 0.18 *($p < 0.0001$)* (Figure 1).

Efflux pump expression in different resistance profile

To determine the expression levels of the *AcrA, ketM, kdeA, kpnEF,* and *kexD* efflux pump genes, we measured the expression levels of these genes between ESBL and carbapenemase producing isolates which were compared with *K. pneumoniae* ATCC 700721. We observed fold change levels of *acrA*, *kdeA*, *ketM*, *kpnEF* and *kexD* as -5,71 ± 2.12, -1.48 ± 1.71, -1.49 ± 1.75, 1.92 ± 2,42 and -3.97 ± 4.00 in ESBL-producing isolates compared to *K. pneumoniae* ATCC 700721,

Table 2. Antibiotic susceptibility profile of *K. pneumoniae* isolates

Strains	Source		Antimicrobial susceptibility										
	Service	Material	CFZ	CXM	CRO	FEP	TZP	IPM	MEM	GEN	AMK	CIP	LVX
ESBL producers													
1	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
2	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
3	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
4	ICU	Sputum	R	R	R	R	S	S	S	S	S	R	R
5	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
6	ICU	Sputum	R	R	R	R	S	S	S	S	S	R	R
7	ICU	Sputum	R	R	R	R	S	S	S	R	S	S	S
8	ICU	Sputum	R	R	R	R	S	S	S	S	S	R	R
9	ICU	Sputum	R	R	R	R	R	S	S	S	S	S	S
10	ICU	Sputum	R	R	R	R	R	S	S	R	R	S	S
11	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
12	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
13	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
14	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
15	ICU	Sputum	R	R	R	R	R	S	S	S	S	S	S
16	ICU	Sputum	R	R	R	R	S	S	S	R	S	R	S
17	ICU	Sputum	R	R	R	R	S	S	S	S	S	S	S
Carbapenemase producers													
18	ICU	Sputum	R	R	R	R	R	R	R	S	S	S	S
19	ICU	Sputum	R	R	R	R	R	R	R	S	S	S	S
20	ICU	Sputum	R	R	R	R	R	R	R	R	S	S	S
21	ICU	Sputum	R	R	R	R	R	R	R	R	S	S	S
22	ICU	Sputum	R	R	R	R	R	R	R	R	R	S	S
23	ICU	Sputum	R	R	R	R	R	R	R	R	S	S	S
24	ICU	Sputum	R	R	R	R	R	R	R	S	S	R	R
25	ICU	Sputum	R	R	R	R	S	R	R	S	S	R	R
26	ICU	Sputum	R	R	R	R	S	R	R	R	R	S	S
27	ICU	Sputum	R	R	R	R	R	R	R	S	S	R	R
28	ICU	Sputum	R	R	R	R	R	R	R	S	S	R	S
29	ICU	Sputum	R	R	R	R	S	R	R	R	S	R	S
30	ICU	Sputum	R	R	R	R	S	R	R	S	S	R	R
31	ICU	Sputum	R	R	R	R	R	R	R	S	S	R	R
32	ICU	Sputum	R	R	R	R	R	R	R	S	S	R	R
33	ICU	Sputum	R	R	R	R	R	R	R	R	S	S	S

CFZ, cefazolin; CXM, cefuroxime; CRO, ceftriaxone; FEP, cefepime; TZP, piperacillin-tazobactam;IPM, imipenem; MEM, meropenem; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; LVX, levofloxacin; S, susceptible; R, resistant; ESBL, extended-spectrum β-lactamase; ICU, intensive care unit.

respectively. Meanwhile, the fold change levels of *AcrA*, *kdeA*, *ketM*, *kpnEF* and *kexD* in carbapenemase-producing isolates were observed as -3,60 ± 1,01, 1,22 ± 0,96, 0,80 ± 1,00, 1.55 ± 0,99 *and* -4.01 ± 5.91 compared to *K. pneumoniae* ATCC 700721, respectively. mRNA expressions of *AcrA, kdeA, ketM* and *kpnEF* in carbapenemase-producing isolates were increased compared to ESBL producing isolates. Moreover, no significant difference was found for *kexD* between ESBL and carbapenemase producing isolates (Figure 2A-E).

Discussion

It is obvious that antibiotics, the most important treatment for bacterial infections, play an important role in medicine. However, infections caused by multidrug-resistant bacteria have been increasing and inevitably incurable diseases will occur in our world [19]. Today, ESBL- and carbapenemase-producing *K. pneumoniae*

Figure 1. Biofilm formation of clinical *K. pneumo*niae isolates. Cells were incubated for an overnight and biofilm formation was determined according to their optical density (OD) values at 595 nm. A significant increase in ESBL producers than carbapenemase producers. Carbapenemase-producing isolates form less biofilm than ESBL-producing isolates. Data are expressed as means ± SD; ESBL producers (n = 17), carbapenemase-producing isolates (n = 16) ***, *p < 0.001*.

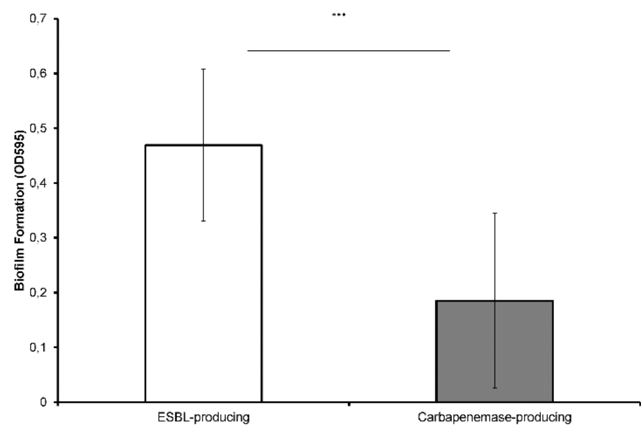
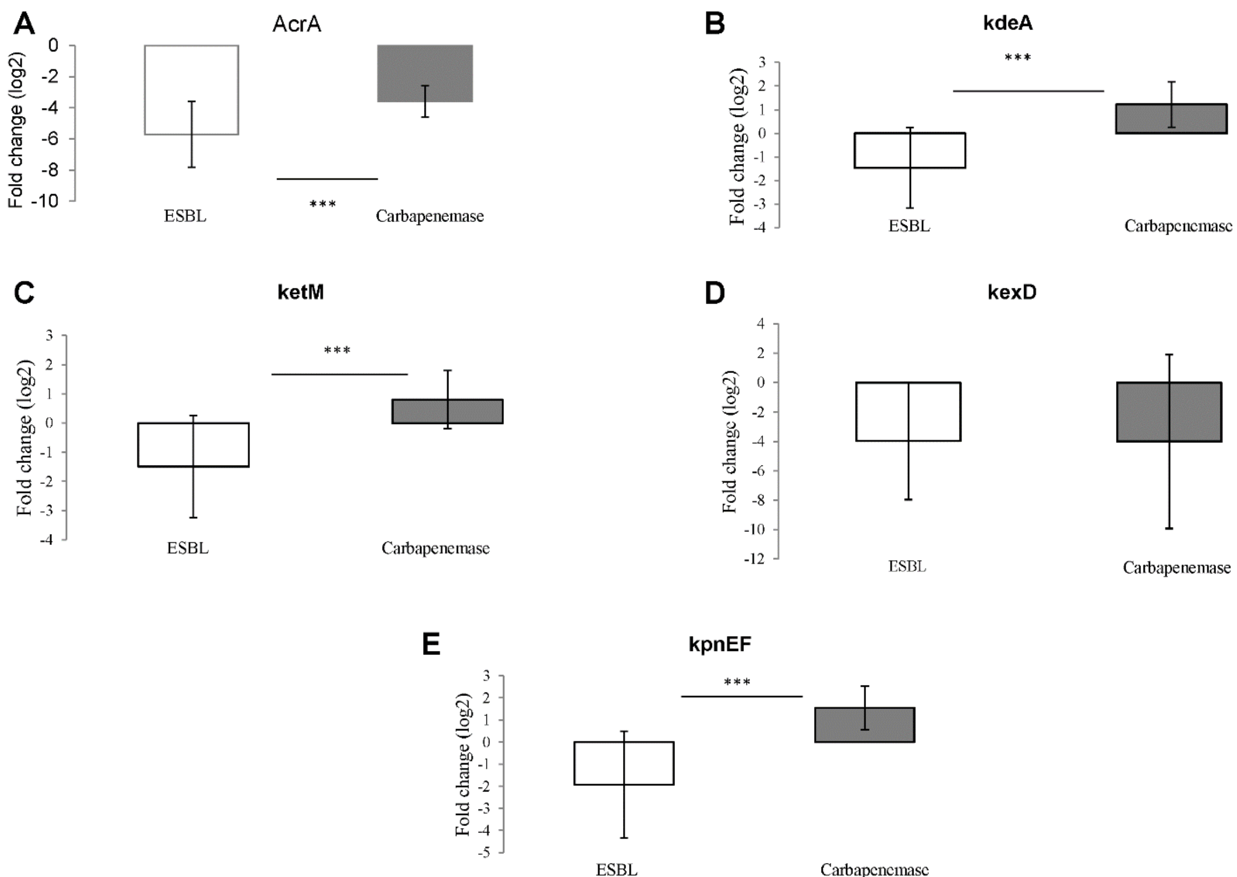


Figure 2. Fold change (log2) of *AcrA* (A), *kdeA* (B), *ketM* (C), *kexD* (D), and *kpnEF* (E) in *K. pneumoniae* isolates. Isolates were divided into two group; ESBL- and carbapenemase-producing isolates. A significant expression observed in carbapenemase-producing isolates than ESBL-producing isolates. No significant difference was found between non-producing and carbapenemase-producing isolates for *kexD* (D) in two groups. Data are expressed as means ± SD; ESBL producers (n = 17), carbapenemase-producing isolates (n = 16) ***, *p < 0.001*.



strains have become a major problem worldwide [20,21]. In this study, we aimed to determine antibiotic susceptibility profile, biofilm formation and efflux pump expression in a total of 33 *K. pneumoniae* isolated from ICU patients.

Previous studies have shown that the prevalence rate of ESBL- and carbapenemase-producing *K.pneumoniae* isolates can be varied from 19% to 60% in different countries [22-25]. To support this result, in our study, 51% of collected isolates were ESBL-producers while 49% of them were carbapenemase-producers. Such different rates of *K. pneumoniae* suggests that the prevalence of ESBL- and carbapenemase-producing isolates are growing rapidly and is related to the excessive use of this class of antibiotics.

Our findings showed that the highest resistance rates were observed for cefazolin, cefuroxime, ceftriaxone and cefepime (100%) and resistance rates to imipenem and meropenem were 48%. However, decreased resistance rates were observed to piperacillin-tazobactam (45%), ciprofloxacin (36%), gentamicin (30%), levofloxacin (27%), and amikacin (9%). Several studies have shown that *K. pneumoniae* isolates are resistant to antibiotics such as third-generation cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides [26,27]. Low-level resistance to amikacin and gentamicin may be good news in the *K.pneumoniae*-related infections for our isolates and furthermore, ciprofloxacin and piperacillin-tazobactam resistance is in line with a recent study, 29.9% and 22.3%, respectively [28].

Biofilm formation is an important virulence factor in many species and biofilms can be difficult to eradicate when associated with diseases such as cystic fibrosis, UTIs, and periodontal diseases [29]. In our study, we divided our isolates into two groups; ESBL-producing and carbapenemase-producing isolates based on their enzyme-mediated resistance profile. Our study revealed that ESBL-producing isolates formed more biofilm than non-producing isolates. Our results are in line with other studies which PER-1 positive isolates produce more biofilm than PER-1 negative ones [30]. Moreover, Gharrah *et al* [31] indicated that ESBL producer strains form more biofilm than non-ESBL producers in *K.pneumoniae*. The uptake of ESBL carrying plasmids seemed to trigger the virulence factor by upregulation of some genes or adding new virulence genes such as type 1 and type 3 fimbriae resulting in greater invasion and biofilm forming abilities in *K.pneumoniae* [32]. Interestingly, our findings showed that carbapenemase-producing isolates formed less biofilm than ESBL-producing and non-producing isolates. Recent studies demonstrate different results, carbapenemase producers *P.aureginosa* and *A. baumannii* formed stronger biofilms than carbapenemase negative isolates [33-35] and an inverse relationship between imipenem-meropenem resistance and biofilm formation [36,37]. These different results can be explained by the clonality (or hetrogeneity) of the carbapenemase-producing isolates [33]. Many more studies are needed to display crosstalk between carbapenemase production and biofilm formation.

To determine whether the overexpression of *acrA, kdeA, ketM, kpnEF,* and *kexD* genes was correlated with plasmid-mediated resistance, we used qRT-PCR to analyse the level of expression of chromosomal-encoded *AcrA, kdeA, ketM, kpnEF,* and *kexD* in 33 selected *K. pneumoniae* clinical isolates. qRT-PCR analyses showed significantly higher expression of *AcrA, kdeA, ketM*, and *kpnEF* in none producers than ESBL producers (data not shown). Thus, the increased expression of these genes did not contribute to resistance against β-lactam antibiotics. Piddock et al [10] also showed that such an increase doesn't always result in clinical level of antibiotic resistance. These results provide the evidence that the main resistance mechanism for β-lactam antibiotics is β-lactamases [38]. It seems that specialized pump systems that contribute antimicrobial resistance are not present, mRNA expression *AcrA, kdeA, ketM* and *kpnEF* in carbapenemase-producing isolates was increased compared to ESBL producing isolates. Moreover, no significant difference was found for *kexD* between ESBL and carbapenemase producing isolates (Figure 2A-E). This result can be supported by MBL (metallo beta-lactamase) producers, overexpressing MexXY-OprM efflux pump in *P.aureginosa* [39]. Our results support the hypothesis that increased combined action of carbapenemase and a secondary resistance mechanism such as increased efflux pump expression can confer resistance to carbapenems [40].

Conclusion

In summary, our findings demonstrated that ESBL production could be linked to biofilm formation together with antibiotic resistance. Moreover, ESBL-producing isolates had decreased efflux pumps' expression compared with carbapenemase producers. These findings suggested that the difference in biofilm formation and efflux pump expression varies amongst bacteria with different resistance profiles.

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Authors' contributions

BY, İT, and TY designed research, performed qPCR experiments, analyzed data and wrote the article; RG and ÇK collected bacterial isolates, conducted phenotypic detection of ESBL production and antimicrobial susceptibility profile. All authors performed critical revision of the manuscript and gave final approval of the submitted version.

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