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Molecular detection of Extended-Spectrum β-lactamases (ESBLs) and biofilm formation in uropathogen *Klebsiella pneumoniae* in Iran

Elham Haghighifar[1], Fatemeh Norouzi[2], Razie Kamali Dolatabadi[1]*

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Abstract

Background: Uropathogenic *Klebsiella pneumoniae* is one of the well-kown uropathogens that have the main rule in biofilm formation. Increased prevalence of ESBL enzyme is one of the therapeutic problems. However, the aims of this study were to characterize the ability of biofilm formation and ESBL-producing isolates produced by urinary tract infection's *K. pneumoniae* to identify the prevalence of this type of infection in the studied area.

Methods: Between the 500 nonrepetitive clinical isolates, 128 isolates were detected as *K. pneumoniae*. Biofilm production of these isolates was showed by Merrit and Christensen method. The standard Kirby-Bauer disk diffusion method was used for antimicrobial susceptibility testing. The phenotype ESBL was confirmed by double disc synergy test (DDST). Genotypic identification of ESBLs did by molecular detection. The statistical analysis was done using software IBM SPSS Statistics (SPSS Inc) and chi-square and Fisher exact tests.

Results: The result of microtiter plate was observed and it was found that 86 (67.2%) isolates had weak biofilm, 24 (18.8%) moderate biofilm, and 18 (14.1%) strong biofilm. Also, 57 (44.5%) out of 128 isolates were diagnosed as MDR. The highest frequency of resistance was identified for cefotaxime 60 (46.9%) and tetracycline 60 (46.9%), and the lowest rate was for amikacin 16 (12.5%). The results of DDST showed 55 of 128 (43%) produced ESBL enzymes. PCR detection in ESBL-producing isolates showed contained bla_{TEM} 33 of 55(63.1%), and bla_{VEB} 13 of 55 (23%). Also, 1 of 55 (2%) had both bla_{TEM} and bla_{VEB}. Also, 5 of 13 (38.4%) isolates that had the bla_{VEB} gene were also MDR and had weak biofilm (8/13; 61.5%), intermediate biofilm (3/13; 23%), and strong biofilm (2/13; 15.4%).

Conclusion: To decrease treatment complications and mortality rate of drug-resistant bacterial infections, rapid detection of β-lactamases genes and evaluation of these properties and infection management programs can help to prevent the transmission of drug resistant-strains.

Keywords: Extended-spectrum β-lactamases (ESBLs), Biofilm Formation, *Klebsiella pneumonia*, Antibiotic resistance, bla_{TEM}, bla_{VEB}

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Introduction

Urinary tract infection (UTI) covers a wide range of asymptomatic microbial colonization to inflammation and widespread microbial invasion into the urinary tract (1). One of the main of UTI morbidity and mortality is the infection in the hospital as a nosocomial infection that is said to account for 25% to 40% of nosocomial infections (2).

Klebsiella pneumoniae is a gram-negative and facultative anaerobic bacteria that is capable of causing a wide range of health care-associated infections (3). Drug

Corresponding author: *Razie Kamali Dolatabadi, r.kamali@resident.mui.ac.ir*

1. Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
2. Department of Microbiology, School of Medicine, Fasa University of Medical Sciences, Fasa, Iran

↑What is "already known" in this topic:
Klebsiella pneumoniae is one of the well-kown uropathogens. The enzyme β-lactamase is the major cause of resistance to betalactam antibiotics in gram-negative bacteria.

→What this article adds:
The findings of this study could help select the appropriate antibiotic treatment. These findings suggest that genes involved in antibiotic resistance are increasing.

resistance is a major problem in almost all countries. Antibiotic multidrug resistant (MDR) in *K. pneumoniae* is conferred primarily by extended spectrum β-lactamase (ESBL), which are enzymes that hydrolyze the β-lactam ring of β-lactam antibiotics (4).

Broad-spectrum beta-lactamase microbial resistance was first observed in early 1980s in Europe and then in the United States shortly after the entry of the third-generation cephalosporins into the treatment line (5). In gram-negative bacteria, the main reason for resistance to beta-lactam antibiotics is the enzyme β-lactamase. Today, there is an increase outbreak and dissemination of ESBL enzymes that hydrolyze and cause resistance to oxyimino-cephalosporins and aztreonam (6). More than 300 different ESBL types are known. However, temoneira (TEM) and sulphydryl variable (SHV) types are widespread ESBLs and CTX-M ESBL is rapid spread (7). TEM, SHV, and OXA were predominant enzyme types until the late 1990s (8, 9). New types that have emerged today consist of families of pseudomonas extended-resistance (PER) and Vietnamese extended-spectrum beta–lactamase (VEB) (10). VEB and PER are rare species and their study is valuable. However, the virulence factors expression, such as genes involved in biofilm production and virulence, plays an significant role in treatment failure among infections caused by highly resistant *K. pneumoniae* isolates (11). Several virulence genes expressions have been related to the persistence and increased survival of *K. pneumoniae* within the host; they consist of quorum sensing genes (12) and genes involved in biofilm formation, which eventually lead to improvement of drug resistance (13, 14).

Biofilm formation is a transitional state of planktonic life, characterized by the adherent accumulation of small bacterial colonies surrounded by extracellular polysaccharide matrix, in which cells are irreversibly attached to various surfaces, including medical devices and damaged tissues (15).

The aims of this study were to characterize the ability of biofilm formation and ESBL-producing *K. pneumoniae* isolates and identify the types of ESBL produced by urinary tract infections *K. pneumoniae* from a large university hospital.

Methods

The 500 nonrepetitive clinical isolates were collected from a university hospital in Isfahan, Iran, from March 2017 to April 2018. Also, written informed consent forms were taken from either the patients or their authorized representatives. This study was approved by the research ethics committee of Isfahan University of Medical Sciences. A total of 128 isolates from urine samples were initially identified as *K. pneumoniae* using the standard IMVIC biochemical tests (indole, methyl red, Voges-Proskauer, citrate) and urease. Confirmation of isolates was done by molecular detection of the ureD gene (243 bp), which hydrolyzed the urea (5). *K. pneumoniae* ATCC 700603 was used as the positive control. The primers were used as listed below:

ure –D F: 5_-CCCGTTTTACCCGGAAGAAG - 3

ure –D R: 5_ - GGAAAGAAGATGGCATCCTGC -3

Biofilm Production (by microtitre plates)

Biofilm production was performed by a modification of the procedure illustrated by Merrit et al (2005) and Christensen et al (1985) (16, 17). The isolates were grown in Brain Heart Infusion Broth supplemented with 2% glucose and sucrose overnight at 37·C° for 24 hours. The cultures were diluted and the cell suspension was used to inoculate sterile flat-bottomed 96-well polystyrene microtitre plate at 37·C° for 48 hours. After 48 hours, the suspension was poured off and the wells were washed 3 times gently. The dried wells were stained with 0.1% crystal violet solution for 20 minutes. Then, the wells were washed 3 times and allowed to dry. A strong biofilm formation can be observed stained layer adhered to the inner wall of the microtitre plate wells.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done using the standard Kirby-Bauer disk diffusion procedure on the Mueller-Hinton agar (Merck). Antibiotic discs were used for the early detection of ESBLs production in *K. pneumoniae* isolates. The guidelines from the Clinical Laboratory Standards Institute (CLSI, 2018) were applied for performance and interpretation (18).

The concentrations of antimicrobial agents used on the Mueller-Hinton agar were as follows: imipenem (10 µg), tetracycline (30 µg), trimethoprim (1.25 µg) sulfamethoxazole (23.75 µg), ceftriaxone(10 µg), cefotaxime(10 µg), amikacin(30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), cefepime (30 µg), and nitrofurantoin (10 µg) (American Bidi). *E. coli* ATCC 25922 was used as a negative control (19).

ESBLs Screening and Phenotypic Identification by DDST

In early screening, detected resistant or decreased sensitivity to more than one of the third generation cephalosporins (3GC-ceftazidime, cefotaxime) were considered as probable ESBL producers (5). The phenotype identification was performed by Double Disc Synergy Test (DDST) on the Mueller-Hinton agar. Antibiotic discs of ceftazidime and ceftazidime-clavulanic acid were placed 15 mm apart and the plates were placed under aerobic incubation at 37 °C. An increase of more than 5 mm in zone diameter for either antimicrobial agent tested in combination with clavulanic acid over the initial zone diameter of the agent assayed was defined as ESBL (18). *K. pneumonia* ATCC700603 and *E. coli* ATCC 25922 were used as a positive and negative control, respectively.

ESBLs Genotypic identification

The *K. pneumoniae* DNA template was extracted from freshly cultured isolates by suspending colonies in Distilled water 2 times and boiling it for 10 minutes. The samples were cooled for 5 minutes at -20°C and then centrifuged for 10 minutes at 10000 rpm (9). The

Table 1. Primers Used for Amplification of ESBLs Genes

Primer	Nucleotide Sequences	Expected Size	PCR Conditions	Reference
TEM-F	TTTCGTGTCGCCCTTATTCC	403	94°C, 3 min; 35 cycles of	34
TEM -R	ATCGTTGTCAGAAGTAAGTTGG		94°C, 45sec, 58°C, 30 sec, 72°C, 60 sec,72°C, 6min	
VEB-F	CGACTTCCATTTCCCGATGC		95°C, 7min; 35 cycles of 94°C, 60 sec, 58°C, 60 sec,	35
VEB-R	GGACTCTGCAACAAATACGC	585	72°C, 2 min,72°C, 5min	

supernatant was applied as the DNA template in polymerase chain reaction (PCR) analysis. The isolates were identified as ESBL by molecular detection of *bla*$_{TEM}$, *bla*$_{VEB}$. The primers of these genes are listed in Table 1. A PCR reaction tube was done in 30 µl mixture of 3 µL 10X buffer, 1 µL of 10 mM $MgCl_2$, and 0.25 µL of 5 U/µL Taq DNA polymerase (Fermentas, Lithuania), 0.5µL each of 10 mM deoxynucleaotidetriphosphates, 1 µL each of 10 µM primers, and 5 µL of plasmid extract in a thermal cycler (Kyratec).

Data Analysis

Data were analyzed via Statistical Package for the Social Sciences (SPSS) version 24 (SPSS Inc). The relation between biofilm production and the amount of biofilm production with drug resistance phenotypes of *K. pneumoniae* was determined by chi-square and Fisher exact tests. The analysis was detected with a confidence level of 95% and $p<0.05$ was considered statistically significant.

Results

The 128 clinical isolates of *K. pneumoniae* were detected by biochemical tests. Molecular identification was performed by PCR of ure D gene and all isolates were positive. Among 128 *K. pneumonia* clinical isolates, 76 (59.3%) were from males and 52 (40.6%) were from females. The result of microtiter plate was 128 (100%) observing biofilm production. Also, 86 (67.2%) isolates had weak biofilm, 24 (18.8%) moderate biofilm, and 18 (14.1%) strong biofilm (Table 2). In this study, MDR profile was considered resistance to at least 3 classes of antimicrobial agents. A total of 57 isolates (44.5%) were identified as MDR.

The Pattern of Resistance to Antimicrobial agents is shown in Figure 1.

Our results indicated that (55/128; 43%) *K. pneumoniae* isolates were producing ESBL. Our results indicated the highest frequency of resistance was demonstrated for cefotaxime 60 (46.9%) and etracycline 60 (46.9%) and the lowest rate was for amikacin 16 (12.5%). We further surveyed the correlation of the MDR profile of the isolates with the ability to form biofilm. Comparison of the drug resistance profile with their biofilm strength index revealed that in MDR isolates, 37 (65%) were weak and 12 (21%) were moderate, while the strong biofilm formers were only 8 (14%). In statistical studies, it was observed that the strains that were resistant to ciprofloxacin and tetracycline were significantly correlated with biofilm production, with a probability value of 0.037 and 0.021.

The results of DDST of *K. pneumoniae* isolates showed that 55 of 128 (43%) isolates produced ESBL enzymes. Molecular detection showed that ESBL-producing *K. pneumoniae* isolates had 63.1% *bla*$_{TEM}$ (35/55) and 23% *bla*$_{VEB}$ (13/55); also, 1 of 55 (2%) had both *bla*$_{TEM}$ and *bla*$_{VEB}$ (Fig. 2). Moreover, 5 of 13 (38.4%) isolates that

Table 2. Biofilm Formation in *K. pneumonia*

Biofilm	Frequency	%
Weak	86	67.2
Intemediate	24	18.8
Strong	18	14.1
Total	128	100

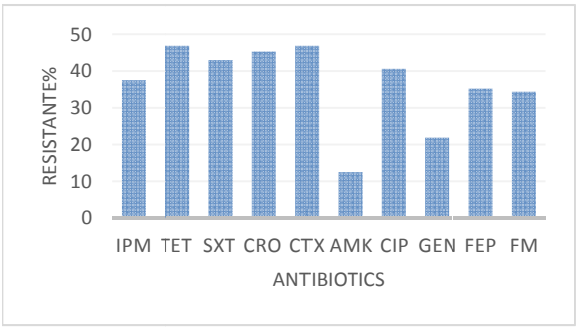

Fig. 1. Pattern of Resistance to Antimicrobial Agents Among all *K. pneumonia* Strains
[a] Abbreviations: IPM, Imipenem; TET, Tetracycline; SXT, Trimethoprim/sulfamethoxazole , Ceftriaxone,CRO; Cefotaxime, CTX; Amikacin,AMK; Ciprofloxacin,CIP; Gentamicin,GEN; Cefepime,FEP; Nitrofurantoin, FM.

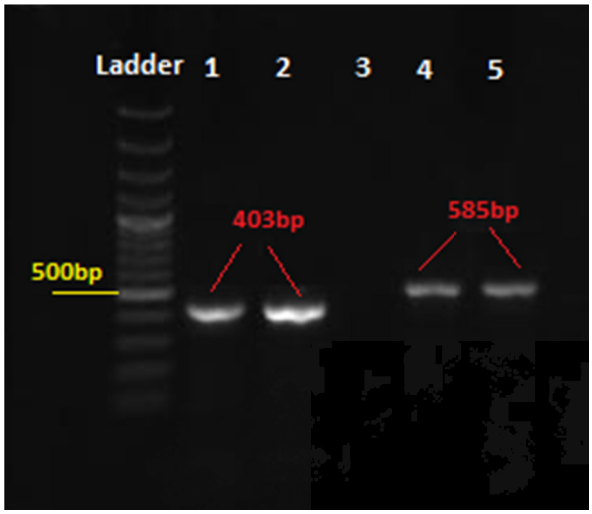

Fig. 2. Polymerase Chain reaction Amplification of bla$_{VEB}$ and bla$_{TEM}$.
Lanes 1: positive control bla$_{TEM}$, Lanes 2: PCR product of bla$_{TEM}$(403 bp), Lane 3: Negative control , Lane 4: positive control bla$_{VEB}$, Lanes 5: PCR product of bla$_{VEB}$ (585 bp).

had the bla_{VEB} gene were also MDR with weak biofilm (8/13; 61.5%), intermediate biofilm (3/13; 23%), and strong biofilm (2/13;15.4%). Also, 14 of 33 (42.4%) isolates that had the bla_{TEM} gene were also MDR withweak biofilm (24/33; 72.7%), intermediate biofilm (5/33;15%), and strong biofilm (4/33;12.12%). There was a significant relationship between the presence of bla_{VEB} gene and cefepime resistance. Also, based on the Pearson chi-square test results, there was an important association between the presence of bla_{TEM} gene and resistance to ceftriaxone and cefotaxime.

Discussion

Recently *K. pneumoniae* resistance to beta-lactam antibiotics has become a worldwide problem in the field of health care. Increased multidrug-resistant *K. pneumoniae* as a serious nosocomial pathogen in patients with urinary tract infection is an important and significant issue. Nowadays, removing MDR UTI is very challenging because not only the hard treatmenting strains are growing but also the antimicrobial resistance is developing (21, 22). Therefore, recognition of the contribution of those strains in infectivity hospitalized patients, in addition to continuous tracking of involved genes in each region, can significantly contribute to the prevention and control of these infections (23). We additionally present document proposed that the MDR profile of *K.pneumoniae* is not related to the biofilm strength. In our study, the highest frequency of resistance was to cefotaxime (46.9%) and tetracycline (46.9%) that was lower than those reported from other Iranian cities by Azizian et al from Hamedan in 2019 (50%), Eghbalpoor et al from Tehran in 2019 (63.3%), Sara Kootia et al from Shiraz in 2019 (50%), and Jahromy et al from Tehran in 2019 (100%) (24-26). In this study, the most effective antibiotic was amikacine with 87.5% sensitivity that is different from other studies, including those of Hossein Forouzandeh (2019) (71.6%) and SajjadYazdansetad (2019) (north-central Iran) (50%). This may be due to the optimal management of infection control at the hospital under study in this study (27, 28). Many bacterial chronic infections due to biofilm formation are difficult to deal with and remove. Biofilm production in *K. pneumoniae* increases colonization and leads to increased UTI. Treatment of these infections is difficult because they render multiple drug resistance (29). In the current study, 78% of isolates were capable of forming biofilms, while in another study in patients with catheter-associated UTI, 89.5% of isolates produced biofilm and among those patients without catheter-associated UTI none produced biofilm (30). In this study, we did not find an association between drug resistance profile and biofilm formation ability, although Diago-Navarro et al reported a correlation within the *K. pneumoniae* isolates causing blood infections (31). In the present study, the strains were resistant to ciprofloxacin, and tetracycline was significantly correlated with biofilm by production, with a probability value of 0.037 and 0.021. The cause of this phenomenon in a *Caenorhabditis elegans* model was shown by Bialek et al between overexpression of efflux pump and increasing virulence (32). Our result to detect the prevalence of several β-lactamase in *K. pneumoniae* clinical isolates revealed that there were 43% *K. pneumoniae* isolates. In our study, 43% of *K. pneumoniae* isolates produced ESBL enzymes, 63.1% bla_{TEM}, 23% bla_{VEB}, and 2% bla_{TEM} and bla_{VEB}.While Tahanasab Z, et al reported that 60.4% isolates were ESBL-producers and 81% carried the bla_{TEM} gene (33). Sedighi M, et al reported that the prevalence of bla_{TEM} and bla_{VEB} was 38% and 6%, respectively (34). Bora et al from India showed frequency of 3 β-lactamase genes (bla_{TEM}, bla_{VEB} and bla_{CTX-M}) but found that bla_{TEM} was predominant in *K. pneumoniae* producing β-lactamase (ESBL positive isolates) (77.58%) (35). However, in Asia as well as in this study, bla_{TEM} has become the predominant type of β-lactamase types in *K. pneumoniae* clinical isolates (36).

Conclusion

This study demonstrated the increased considerable bla_{TEM} and bla_{VEB} prevalence of *K. pneumoniae* strains circulating in hospitals in Isfahan, Iran. This MDR incremental trend is correlated with the presence of bla_{TEM}. The high prevalence of drug resistance genes indicates the necessity for serious monitoring of antibiotic administration to control infections caused by *Enterobacteriaceae* in Iran. However, to decrease complications of treatment and mortality rate for drug-resistant bacterial infections, rapid diagnosis of β-lactamases genes and evaluation of these properties could help to prevent transmission of drug resistant-strains and infection management programs. Also, further studies are required to get gather more detailed and impressive results.

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Conflict of Interests

The authors declare that they have no competing interests.

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