

**ORIGINAL ARTICLE****Correlation between biofilm formation and carbapenem resistance among clinical isolates of *Klebsiella pneumoniae*****Hossein Ali Rahdar<sup>1,2</sup>, Ebadallah Shiri Malekabad<sup>1</sup>, Ali-Reza Dadashi<sup>3\*</sup>, Elahe takei<sup>4</sup>, Masuod Keikha<sup>5</sup>, Hossein Kazemian<sup>6</sup>, Morteza Karami-zarandi<sup>4</sup>****OPEN ACCESS**

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**ABSTRACT**

**Background:** *Klebsiella pneumoniae* is a Gram-negative enteric bacterium that causes nosocomial infections; this bacterium has survived from harsh condition using biofilm formation in hospital equipment and cause severe infection. In the other hand, the emergence and extension of carbapenem resistance burden among *K. pneumoniae* producing biofilm is the current concern of public health services. There are controversial findings about this subject. The aim of this study was to evaluate the correlation between biofilm formation and resistance to carbapenem among clinical isolates of *K. pneumoniae*.

**Methods:** A total of 160 *K. pneumoniae* isolates were collected from various infections of hospitalized patients. The Carba NP test and molecular methods were used for detection of carbapenem resistance isolates of *K. pneumoniae*. Subsequently, the ability for biofilm production was performed from all isolates. Finally, Correlation of biofilm formation among carbapenem resistant isolates was calculated using  $\chi^2$  and Fisher's exact tests.

**Results:** Among *K. pneumoniae* isolates 42.5% have carbapenemase activity by Carba NP test, while carbapenemase genes were detected in 35.6% of isolates in amplification assay. Moreover, there are 52.5% (n= 84) of all isolates were formed a strong biofilm, while 38.1% (n= 61) and 9.3% (n= 15) of isolates were middle and weak biofilm producer, respectively. Among carbapenem resistant cases (n= 68), there are 77.9% (n= 53) and 22% (n= 15) of isolates were reported as strong and middle biofilm producer, respectively. We see a significant correlation was seen between biofilm formation ability and carbapenem resistant isolates (p-value < 0.00001).

**Conclusion:** The increase of carbapenem resistance burden in biofilm producing isolates of *K. pneumoniae* is considered as serious alert and the basic measures to combat this phenomenon is imperative.

**Keywords:** *Klebsiella pneumoniae*, Biofilm, carbapenemase, carbapenem resistant

## INTRODUCTION

Due to the emerging and increasing antibiotic resistance, treatment of infectious diseases has become a major concern (1, 2). Decreasing speed of discovery of new antimicrobials into the market is led to emerging and rapid spreading of the drug resistant isolates. Hence, spreading of these drug resistant isolates is a serious threat to public health (1-4). Formerly,  $\beta$ -lactam antibiotics have been the main drug of treatment for infectious diseases due to gram negative bacilli, but are facing the antimicrobial resistance scenario (5). For example, extended-spectrum  $\beta$ -lactamase (ESBL)-producing isolates has increased worldwide spread with significant morbidity and mortality (6, 7). In this regard, carbapenems have been introduced for next choice against drug resistant isolates. This antibiotic family has the broadest activity spectra and is still the most active drugs against ESBL producing Enterobacteriaceae (8, 9). Unfortunately, carbapenem-resistant *Klebsiella pneumoniae* strains have been emerging and were spreading worldwide which cause limiting chemotherapy in various infections (10, 11).

*K. pneumoniae* is one of the most common human pathogen which is recovered from a wide range of community and hospital acquired infections such as pneumonia, bacteremia, urinary tract and respiratory tract infections (12). The major virulence factors of *K. pneumoniae* are capsule, fimbriae, lipopolysaccharides (LPS), and biofilm formation (4). Concern about biofilm formation of *K. pneumoniae* are associated with colonization of this bacterium in many medical conditions including indwelling medical devices, upper respiratory tract infections, peritonitis, and urogenital infections (13). The bacterial population can survive in harsh conditions within host, by the formation of biofilm. (14).

Importantly, it has been reported that about 40% of biofilm producing *K. pneumoniae* were recovered not only from urine but also from blood, sputum and wound swabs (15). Specific physiological and genetically interactions within the biofilm are causing a dramatic increase of antimicrobial resistant agents. (16).

Previously studies have shown various associations between antibiotic resistance and

biofilm formation. In this case, some researchers were reported that increased resistance was correlated with high biofilm production (17, 18), while other studies reported that biofilm formation decreased in high resistance isolates (19, 20). Taken together, the association between antibiotic resistance and biofilm formation is currently unclear and remains under investigation (21, 22).

The increasing incidence of ESBL and carbapenemase-producing strains of *K. pneumoniae* in health care facilities is a cause of global concern. This scenario is exacerbated when these strains grow as a biofilm, causing a dramatic increase in the resistance to antimicrobial agents (16). Therefore, the aim of this study was evaluation of correlation between biofilm formation and resistance to carbapenem among clinical isolates of *K. pneumoniae*.

## MATERIALS AND METHODS

**Bacterial Strains:** A total of 160 *K. pneumoniae* isolates was collected from various infections of hospitalized patients between April 2018 and March 2019 at Army hospitals. Identification of bacterial isolates was performed using phenotypic and biochemical tests according to standard methods (23).

**Phenotypic Screening of Carbapenemase Enzymes:** The Carba NP test was used for detection of carbapenemase activity according to standard protocol (24). Briefly, all isolates were grown overnight on Mueller-Hinton agar. One loop of the bacterial colonies was suspended in a 1.5-ml micro tube containing 0.1 ml of 20mM Tris-HCl lysis buffer and mixed very well. This lysate was mixed with 0.1ml of indicator solution consisting of 0.05% phenol red with 0.1 mmol/liter ZnSO<sub>4</sub>, which was previously adjusted to pH 7.8, and 6 mg/ml imipenem and, as a control tube, the phenol red solution without antibiotic. Finally, tubes were incubated at 35°C for 2 h. after that, color change from red to orange/yellow in the antibiotic-containing tube, which was interpreted as a positive result. *K. pneumoniae* ATCC BAA-1705 was used as a positive control (24).

Table 1: Primers used to identify carbapenem resistant genes

ESBLs <sup>1</sup>	Primer	PCR product	Reference
IMP	Imp-F 5'-GGA ATA GAG TGG CTT AAY TCT C-3' Imp-R 5'-CCA AAC YAC TAS GTT ATC T-3'	188	(13)
VIM	Vim-F 5'-GAT GGT GTT TGG TCG CAT A-3' Vim-R 5'-CGA ATG CGC AGC ACC AG-3'	390	(3)
NDM	NDM-F, 5'-GGTTTGGCGATCTGGTTTTTC-3' NDM-R 5'-CGGAATGGCTCATCACGATC-3'	621	(5)
OXA-48	OXA-48F5'-TTGGTGGCATCGATTATCGG-3' OXA-48R5'-GAGCACTTCTTTTGTGATGGC-3'	743	(4)
KPC-	KPC-1F5'-CGTTCTTGTCTCTCATGGCC-3' KPC-1R5'-CCTCGCTGTGCTTGTTCATCC-3'	796	(4)

<sup>1</sup>extended spectrum  $\beta$ -lactamase

*Detection of Carbapenemase-Related Genes:* Chromosomal DNA was extracted using a simple boiling method (25). Polymerase chain reaction was performed using specific primers to detect genes encoding carbapenemase enzymes (*bla*IMP, *bla*VIM, *bla*NDM, *bla*KPC, and *bla*OXA-48-like), as previously described (3). The PCR products were separated by electrophoresis in 1% agarose gel with 1× TBE (Tris/ borate/EDTA) buffer which stained with safe stain load dye (CinnaGen Co., Tehran, Iran) and finally was visualized under ultraviolet illumination.

*Biofilm assay:* Biofilm formation ability of all isolated performed according to the described method previously. Briefly, in 96 cells microplate,

isolates were incubated in Luria broth (LB) at 37°C for 18 h. Then 200  $\mu$ l of TSB was transferred to every well of the sterile microplate, then 10  $\mu$ l of adjusted 0.5 McFarland bacterial suspension was added into the wells and was incubated at 37 °C for 24 h. After incubation, supernatant discharged from the wells and was washed 3 times with saline. Then 200  $\mu$ l of 1% crystal violet was added for 20 min into the wells and was washed 3 times with saline and dried. Finally, 200  $\mu$ l of DMSO was added to each well and the plate was examined by ELISA Plate Reader at 595 nm wavelength. Each test was repeated three times (26).

Table 2. Biofilm formation ability and carbapenem resistant genes

<i>Bla</i> <sup>1</sup> VIM			<i>bla</i> IMP			<i>bla</i> OXA48 like			<i>bla</i> NDM		
Strong	Middle	Weak	Strong	Middle	Weak	Strong	Middle	Weak	Strong	Middle	Weak
29	3	-	7	2	-	11	4	-	1	-	-

<sup>1</sup>*bla*: beta lactamase

The ability to produce biofilm was considered in four categories:

Group 1: strong biofilm OD > 0.5.

Group 2: middle biofilm 0.5 > OD > 0.3.

Group 3: weak biofilm OD < 0.3.

Group 4: Lack of biofilm OD < 0.15.

*Statistical Analysis:* Correlation of biofilm formation among resistant and susceptible isolates was calculated using  $\chi^2$  test and Fisher's exact tests in SPSS software. p-values  $\leq 0.05$  were considered to be statistically significant.

## RESULTS

*Carbapenemase producing isolates:* Among 160 *K. pneumoniae* isolates 42.5% (n: 68) were carbapenemase producer by phenotypic method, while carbapenemase genes were detected in 35.6% (n: 57) of isolates by molecular method. Distribution of carbapenemase genes among carbapenem resistant cases (n: 68) were *bla*VIM 47% (n: 30), *bla*OXA-48-like 22% (n: 15), *bla*IMP 13.2% (n: 9) and *bla*NDM 1.4% (n: 1). *bla*KPC gene was detected in isolates 2/9% (n:2) (Figure 1).

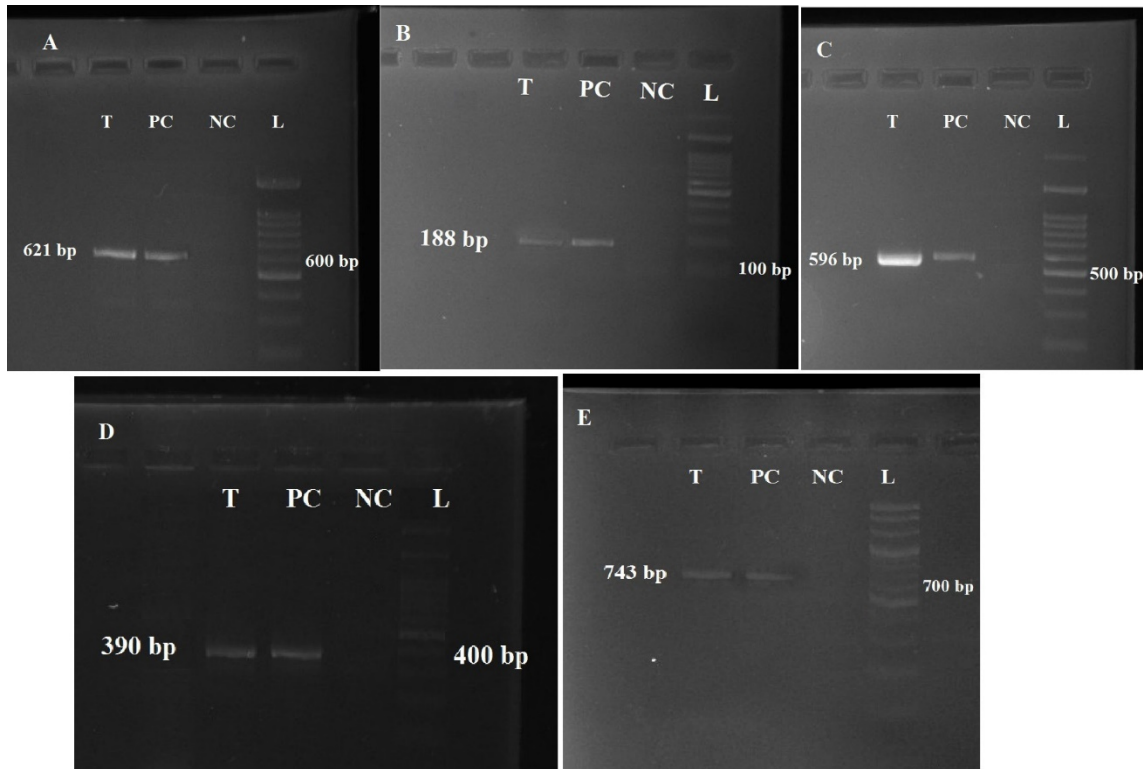


Figure 1: Images related to genotypic study of cluster colonies with carbapenem resistant genes. *Klebsiella pneumoniae* 8053 AO (KPC gene transporter), *Pseudomonas aeruginosa* 510 PO (IMP, VIM), *Escherichia coli* 01 MH (NDM gene carrier), *Klebsiella pneumoniae* 1514 Kp (OXA48 transporter) as positive control.

**Biofilm producing isolates:** According to our results, 52.5% (n: 84) of all isolates were formed strong biofilm, while 38.1% (n: 61) and 9.3% (n: 15) of isolates were middle and weak biofilm producer, respectively. Totally, from 92 carbapenem sensitive cases, 33.6% (n: 31), 50% (n: 46) and 16.3% (n: 15) were strong, middle and weak biofilm producer. But, among carbapenem resistant cases (n: 68), 77.9% (n: 53) and 22% (n: 15) of isolates were reported as a strong and middle biofilm producer, respectively. Also, among carbapenem phenotypical resistant cases (no carbapenemase gene detected) 5 and 6 isolates showed strong and middle biofilm formation ability. A weak biofilm producer has not been seen in carbapenem resistant case. In this regard, a significant correlation had seen between biofilm formation ability and resistance to carbapenem ( $p < 0.00001$ ).

## DISCUSSION

In the present study, the correlation between biofilm formation and resistance to carbapenem among clinical isolates of *K. pneumoniae* was evaluated. According to our results, a frequent gene among carbapenem resistant isolates was *bla<sub>VIM</sub>*, but only one isolates harbored the *bla<sub>NDM</sub>* gene. It has been reported that *bla<sub>VIM</sub>* gene is more frequent than the *bla<sub>IMP</sub>* gene (26). Also, Hosseinzadeh et al. (27), was reported that more than 10% of the isolates carried the *bla<sub>NDM-1</sub>* gene. The *bla<sub>KPC</sub>* gene was not detected in any of our strains.

Interestingly, a significant correlation has been seen between carbapenem resistance and biofilm formation ability of the isolates. Although among carbapenem sensitive isolates, a strong biofilm producer were seen, but majority of carbapenem resistant isolates were strong biofilm producers. In this case, it has been reported that some

correlations exist between biofilm-formation and antibiotic resistance among *K. pneumoniae* strains. For example, among 150 *K. pneumoniae* strains, isolated from sputum and urine, significant association has been seen among biofilm formation with ESBL production (15). Another study was showed that, MDR *K. pneumoniae* strains are form a richer biofilm rather than susceptible ones (28). According to Khodadadian et al in 2018, significant correlation was seen between strong biofilm formation and prevalence of VIM1 and IMP1 genes (26).

In some cases, antibiotic resistance genes are responsible for this phenomena correlation. It has been demonstrated that, resistance genes in special plasmids can regulate biofilm formation in *K. pneumoniae* strains (29).

In conclusion, the acquisition of specific antibacterial resistance can compromise or enhance biofilm formation among the bacterial population. Therefore, the increasing prevalence of drug resistant and biofilm producer *K. pneumoniae*, mostly in hospital associated setting and our data supporting the correlation of biofilm formation with the antibiotic resistance acquisition should alert even more regarding the concern about this pathogen.

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