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The Effect of Antimicrobial Peptide LL-37 on Biofilm Formation of *Klebsiella pneumoniae* Isolated from Catheter-Associated Urinary Tract Infection Patients

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Abstract: Opportunistic pathogen *Klebsiella pneumoniae* Most commonly causes nosocomial infections and particularly affects persons with weakened immune systems. For survival and immune evasion during infection, *K. pneumoniae* makes use of a variety of virulence factors, and caused many infections include pneumonia, bacteremia, UTI. The aim of this research was to assess the antimicrobial peptide's effectiveness against *K. pneumoniae* isolated from patients with UTI as an antibiofilm agent. This study included 170 urine samples which collected from October 2022 to March 2023 from patients with urinary catheters from Baghdad hospitals. The biofilm formation test was carried out for 28 strong biofilm isolates after treatment with LL-37 peptide. The results of biofilm formation after treatment with LL-37 peptide showed an obvious inhibitory activity where the most of isolates under test became ranged from non-biofilm to moderate. Results of five mdr *K. pneumoniae* isolates' lowest inhibitory concentrations (MICs) against LL-37 revealed that the MIC range of LL-37 were (1.9-500 µg/ml), in conclusion, LL-37 has a significant effect on *K. pneumoniae* growth and biofilm formation at very low concentrations.

Keywords: Biofilm formation, *Klebsiella pneumoniae*, LL-37.

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Introduction

The majority prevalent healthcare-associated infection and the initial cause of all later bloodstream infections is catheter-associated urinary tract infection (CAUTI)s (1). It was shown that 80% of nosocomial urinary tract infections and 40% of all hospital-acquired infections are caused by CAUTIs (2). The use of indwelling urinary catheters is anticipated to rise, and a sizable majority of hospital inpatients have one put at some point during their stay. Urinary catheters with plastic coatings undermine the urinary tract's natural defenses and encourage the growth of bacterial colonies or biofilms, which may cause CAUTIs. (3) A member of the *Enterobacteriaceae* family, *Klebsiella pneumoniae* is a Gram-negative bacterial pathogen. It mostly affects those who have impaired immune systems and is frequently linked to nosocomial infections One of the most prevalent organisms causing infections, such as pneumonia, urinary tract infections, and bacteremias, is *Klebsiella pneumoniae*. (5). Where there are many local studies that indicate different resistance mechanisms in Klebsiella bacteria, in addition to their ability to form a strong biofilm (6,7). Pathogenic *K. pneumoniae* may firmly form biofilms and attach to both biotic and abiotic surfaces, shielding the bacteria and promoting their invasive infections, host immune response protection, and AMR. These biofilms are also essential for the colonization of medical devices, which

results in device-associated nosocomial infections, They are thought to make about 25% of all HAIs, or hospital-acquired infections, in acute care hospitals in the United States. In hospitals in Egypt, *Klebsiella spp*. were the pathogens most often isolated from HAIs (8). Initial attachment, irreversible attachment, biofilm growth, biofilm maturity, and biofilm dispersion are all dynamic processes in the production of bacterial biofilms (9). Antimicrobial peptides (AMPs) are thought to represent a novel class of antibacterial compounds. Most experts agree that AMPs target bacterial cell membranes via electrostatic interactions, disrupt the membrane to form holes, and subsequently eliminate the bacterium (10). The wide variety of bactericidal activity of LL-37 as an AMP type is effective against both Gram-positive and Gram-negative bacteria. LL-37 may carry out differentiation and proliferation immunomodulatory mechanisms that promote wound healing. (11).

The purpose of the study is to find out the impact of the LL-37 peptide on the biofilm formation of *klebsiella pneumoniae* among Iraqi patients with catheter-associated urinary tract infection.

Materials and methods

samples collection

Overall 170 urine samples taken from patients in Baghdad hospitals who had urinary catheters implanted and had urinary tract infections between October 2022 and March 2023. The samples were obtained using sterile containers and promptly sent to the microbiology lab at the University of Baghdad's Institute of Genetic Engineering and Biotechnology for Postgraduate Studies.

Isolation and identification of *klebsiella pneumoniae*

The collected samples were cultured on MacConkey agar and CHROME agar media and incubated at 37 °C for 24 h. Biochemical assays were used to identify the colonies on these medium. The results of biochemical tests were confirmed by VITEK-2 system (BioMerieux, France).

Minimum inhibitory concentrations (MIC) of LL-37 peptide

The MIC was calculated using the microdilution technique (Microtiter Plate Assay with Resazurin Dye) the Clinical and Laboratory Standards Institute's suggestion. (12). At doses ranging from 0.4 to 500 g/ml, the LL-37 peptide was diluted in Mueller Hinton Broth (MHB) using a 1:2 serial dilution. Before the test, a *K. pneumoniae* subculture in Brain heart agar was cultured for 18 to 24 hours at 37 °C to create the bacterial inoculum. The bacterial solution was diluted down to $1x10^8$ CFU/mL in order to achieve a turbidity of 0.5 on the McFarland scale. The material was then diluted in MHB 1:2 to a final concentration of $5x10^5$ CFU/mL. The peptides were serially diluted before being added to the 96-well plate that contained the diluted bacterial solution. 100 µl of compound and 100 µl of diluted bacteria suspension made up the final amount of 200 µl per well. In order to perform negative and positive growth controls, *k. pneumoniae* with MHB was added to one line of the wells and merely MHB to the other. Resazurin (0.015%) was added to each well after 24 hours at 37 °C and incubated for 20 minutes to see if the color changed. After incubation, wells that showed no color change (blue resazurin's color stayed constant) were given a score higher than the MIC value. The minimum chemical concentration (MIC) was identified at the conclusion of the incubation period

as the lowest level at which bacterial growth was not seen.

Biofilm formation of *klebsiella pneumoniae* isolates treated with LL-37 peptide

The production of *Klebsiella pneumoniae* biofilm was measured using the microtiter plate method (10). All isolates were cultured in Brain Heart Infusion Broth for one night at 37°C. Each isolate was introduced using a pipette to the Tryptic Soy Broth containing 1% glucose (TSB). The bacterial isolate suspension's turbidity was fixed to McFarland No. 0.5. A sterile 96-well microtiter plate was filled with triplicate cultures of each isolate in a volume of (200 µl). The plate was covered with its lid and incubated for 24 hours at 37°C under aerobic conditions. following the incubation period, to remove bacteria that weren't adhering, the planktonic cells were twice rinsed with phosphate buffer saline (PBS). 200 µl of 100% methanol were used to fix the adhering bacterial cells in each well for 20 min at room temperature. Each well received 200 µl of 0.1% crystal violet, which was then left to stain the adhering cells for 15 minutes. Following the staining process, extra stain was removed by repeatedly washing with distilled water (2-3 times). To ensure full drying, the plate was left to air dry for around 30 minutes at room temperature. Afterwards, 33% acetic acid was used to remove the discoloration. The average of the OD values of the sterile medium was deducted from each test result once it had been calculated. To determine whether or not isolates are biofilm producers, the cut off value (ODc) was calculated (13).

ODc: Average OD of negative control + (3 × standard deviation (SD) of negative control), OD isolate: Average OD of isolate – ODc.

OD ≤ ODc*: Non Biofilm, ODc < OD ≤ 2ODc: Weak, 2ODc < OD ≤ 4ODc: Moderate, OD > 4ODc: Strong.

Results and discussion

Isolation and identification of *klebsiella pneumoniae*

The results of VITEK-2 system, biochemical tests, CHROMagar and MacConkey agar medium identified 61 isolates as K. pneumoniae from all collected bacterial cultures.

Minimum inhibitory concentrations (MICs) of LL-37 against *k. pneumoniae* isolates

Minimum inhibitory doses of the antimicrobial peptide LL-37 for five isolates of *K. pneumoniae* that are mdr were measured using a microtiter plate assay with resazurin dye. The results showed that the inhibitory activity ranged from (1.9-500 µg /ml) and that there were differences in the MICs between the isolates (Figure 1 and table 1). Certain isolates, such Kp55 and Kp6, were barely impacted by concentrations of 1.9 µg /ml and 3.9 µg /ml, respectively, while others, including Kp40 and Kp56, were inhibited by 31.2 µg /m and 15.6 g/ml, respectively. The MICs of isolate kp11 were found to be 62.5 µg /ml.

Table (1): The minimum inhibitory concentrations (MICs) of LL-37 against *Klebsiella pneumoniae* isolates at concentrations (0.4 -500 µg/ml).

LL-37 conc. (µg/ml)	Isolate code				
	Kp11	Kp56	Kp40	Kp6	Kp55
500	-	-	-	-	-
250	-	-	-	-	-
125	-	-	-	-	-
62.5	-	-	-	-	-
31.25	+	-	-	-	-
15.6	+	-	+	-	-
7.8	+	+	+	-	-
3.9	+	+	+	-	-
1.9	+	+	+	+	-
0.9	+	+	+	+	+
0.4	+	+	+	+	+

(- = no growth, + = growth)

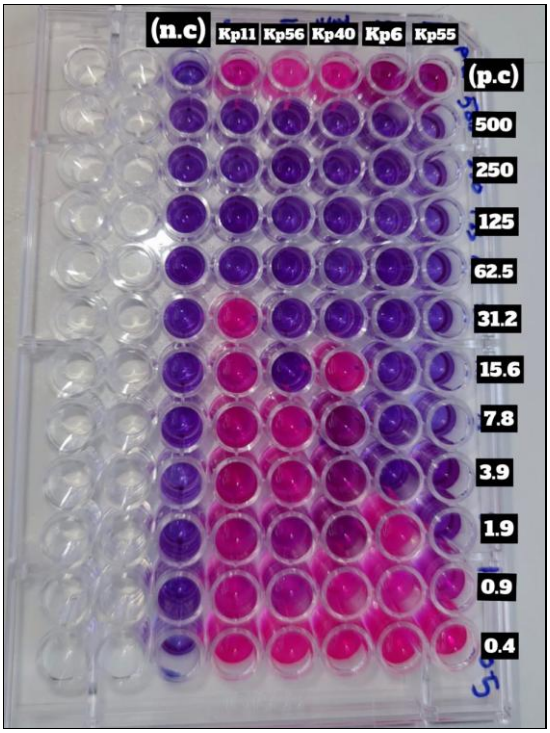

Figure (1): The minimum Inhibitory Concentrations (MICs) of LL-37 at concentrations (0.4 - 500µg/ml) against *Klebsiella pneumoniae* Isolates by Microtiter Plate Assay with Resazurin Dye.

The weak antibacterial activities of LL-37 are sensitive to a wide range of Gram-positive and Gram-negative bacteria, including pathogens from the genera *Pseudomonas*, *Escherichia*, *Staphylococcus*, and *Enterococcus*. LL-37 uses direct antibacterial effects as well as immunomodulation to eliminate germs. The primary mechanism of action is membrane disruption, just as other AMPs (14). Limited studies have been conducted on the antimicrobial effect of the LL-37 peptide, including a study by (15). Very few studies have assessed the importance of biological levels of LL-37 to its antimicrobial activity in vivo, Despite the existence of proof that native or artificial LL-37 exhibits potent antimicrobial activity in vitro against a wide range of pathogens,

including viruses, fungi, and both Gram-positive and Gram-negative bacteria. (16), demonstrated that various LL-37 concentrations were used to incubate the three Kpn isolates (ATCC25955, Col-S, and Col-R). Notably, LL-37 did not appear to have any noticeable effects on any of the isolates at doses lower than 50 g/ml. Bacteria have created a variety of LL-37 resistance mechanisms since AMPs have evolved alongside them over millions of years (17).

Biofilm formation of *klebsiella pneumoniae* isolates treated with LL-37 peptide

Five *K. pneumoniae* isolates treated with sub minimum inhibitory concentration of LL-37 peptide were detected for biofilm formation by microtiter plate (Table 2).

Table (2): Biofilm Strength Determination.

Conc. of LL-37 peptide	OD of Kp55 isolate	Result	OD of Kp40 isolate	Result	OD of Kp56 isolate	Result	OD of Kp11 isolate	Result	OD of Kp6 isolate	Result	Neg. control
125	0.01	Non biofilm	0.15	Weak	0.15	Weak	0.35	Moderate	0.12	Weak	**0.053**
62.5	0.02	Non biofilm	0.16	Weak	0.13	Weak	0.31	Moderate	0.15	Weak	**0.024**
31.2	0.06	Non biofilm	0.19	Moderate	0.15	Weak	0.23	Moderate	0.16	Weak	**0.017**
15.6	0.11	Weak	0.21	Moderate	0.15	Weak	0.27	Moderate	0.16	Weak	**0.031 (AV.) 0.019 (SD.) 0.09(Cut off)**
7.8	0.13	Weak	0.21	Moderate	0.22	Moderate	0.31	Moderate	0.23	Moderate	
3.9	0.22	Moderate	0.27	Moderate	0.22	Moderate	0.74	Strong	0.22	Moderate	
1.9	0.23	Moderate	0.28	Moderate	0.28	Moderate	0.73	Strong	0.27	Moderate	
0.9	0.24	Moderate	0.31	Moderate	0.32	Moderate	0.83	Strong	0.28	Moderate	
0.4	0.24	Moderate	0.33	Moderate	0.32	Moderate	0.98	Strong	0.33	Moderate	

As shown in Table 2, most of the isolates ranged in biofilm formation from Non-biofilm formation to moderate. Antibiofilm Peptides must have a special mode of action that includes the biofilm lifestyle since they may be distinguished from antimicrobial Peptides. Several biofilm growth processes, including as adhesion organelles, matrix components, whether or not biofilms are organized, quorum-sensing mechanisms, and dispersion, are highly variable amongst the many species that antibiofilm peptides target (18). Additionally, a study's findings demonstrated that AMPs' antibiofilm actions are often independent of their antibacterial effects toward planktonic cells (19). Also when the peptide was exploited as an anti-bacterial biofilm on *pseudomonas aeruginosa* by De La Fuente-Núñez (20), Results indicated that biofilm formation was clearly suppressed, with a significant decrease in biofilm height, when biofilm cells of either PAO1 or PA14 were treated with sublethal quantities of the peptide (20 g/ml; 1/15 MIC). Moreover (21),

conducted research, it was established that LL-37's capacity to prevent the development of biofilm, particularly at physiologically appropriate concentrations, is a potential trait for the treatment of chronically infected wounds. Additionally, the LL-37 peptide was applied to regulate biofilm development in a local investigation (22).

Conclusion

The LL-37 peptide's potent antibacterial activity against *Klebsiella pneumoniae* with a robust biofilm suggests that it may be employed as a substitute therapeutic agent for treating isolates of *K. pneumoniae* that are multidrug resistant and responsible for catheter-associated urinary tract infections.

References

1. Werneburg, G. T. (2022). Catheter-Associated Urinary Tract Infections: Current Challenges and Future Prospects. Research and Reports in Urology, 14: 109–133.
2. Kranz, J.; Schmidt, S.; Wagenlehner, F. and Schneidewind, L. (2020). Catheter-associated urinary tract infections in adult patients: Preventive strategies and treatment options. Deutsches Ärzteblatt International, 117(6): 83.
3. Venkataraman, R. and Yadav, U. (2023). Catheter-associated urinary tract infection: An overview. Journal of Basic and Clinical Physiology and Pharmacology, 34(1): 5–10.
4. Ali, S.; Alam, M.; Hasan, G. M. and Hassan, M. I. (2022). Potential therapeutic targets of *Klebsiella pneumoniae*: a multi-omics review perspective. Briefings in Functional Genomics, 21(2): 63–77.
5. Stojowska, K.; Łupkowska, A.; Kuczyńska- wiśnik, D. and Laskowska, E. (2021). Antibiotic Heteroresistance in *Klebsiella pneumoniae*. International Journal of Molecular Sciences 2022, 23(1): 449.
6. Hamad, S. T.; Ghaim, K. K. and Al-lawi, A. A. (2022). Prevalence of Carbapenemase Genes in Klebsiella pneumoniae Isolates from Patients with Urinary Tract Infections in Baghdad Hospitals. Iraqi Journal of Biotechnology, 21(1):102-114.
7. Ghaima, K. K. and Khalid, T. M. (2022). Molecular Detection of acrAB and oqxAB Genes in *Klebsiella pneumoniae* and Evaluation the Effect of Berberine on their Gene Expression. Iraqi Journal of Biotechnology, 21(2):124-135.
8. Zaki, B. M.; Fahmy, N. A.; Aziz, R. K.; Samir, R. and El-Shibiny, A. (2023). Characterization and comprehensive genome analysis of novel bacteriophage, vB_Kpn_ZCKp20p, with lytic and anti-biofilm potential against clinical multidrug-resistant *Klebsiella pneumoniae*. Frontiers in Cellular and Infection Microbiology, 13, 3.
9. Zhu, T.; Yang, C.; Bao, X.; Chen, F. and Guo, X. (2022). Strategies for controlling biofilm formation in food industry. Grain & Oil Science and Technology, 5(4): 179–186.
10. Browne, K.; Chakraborty, S.; Chen, R.; Willcox, M. D.; Black, D. S.; Walsh, W. R., *et al*. (2020). A new era of antibiotics: the clinical potential of antimicrobial peptides. International Journal of Molecular Sciences, 21(19): 7047-7070.
11. Lai, L.; Zou, W.; Zhang, Y.; Tu, Y.; Li, S.; Xin, T., *et al*. (2022). Multifunctional MIL-101 nanoparticles with Fenton-like reactions to Co-deliver LL-37 peptide and Vancomycin for targeted NIR imaging and Drug-resistant bacteria treatment. Chemical Engineering Journal, 435: 135084-135099.
12. Clinical and laboratory standards institute (CLSI). (2021). performance standards for antimicrobial susceptibility testing. Second informational supplement. CLSI document M100-S22. Clinical and Laboratory Standard.
13. Coffey, B. M. and Anderson, G. G. (2014). Biofilm formation in the 96-well microtiter plate. Methods in Molecular Biology, 1149: 631–641.
14. Wang, G.; Mishra, B.; Epand, R. F. and Epand, R. M. (2014). High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1838(9): 2160-2172.
15. Krasnodembskaya, A.; Song, Y.; Fang, X.; Gupta, N.; Serikov, V.; Lee, J. W., *et al*. (2010). Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial

peptide LL-37. Stem cells, 28(12): 2229-2238.

16. Al-Farsi, H. M.; Al-Adwani, S.; Ahmed, S.; Vogt, C.; Ambikan, A. T.; Leber, A., *et al*. (2019). Effects of the antimicrobial peptide LL-37 and innate effector mechanisms in colistin-resistant Klebsiella pneumoniae with mgrB insertions. Frontiers in Microbiology, 10: 2632.
17. Ridyard, K. E. and Overhage, J. (2021). The potential of human peptide LL-37 as an antimicrobial and anti-biofilm agent. Antibiotics, 10(6): 650-682.
18. Pletzer, D. and Hancock, R. E. (2016). Antibiofilm peptides: potential as broad-spectrum agents. Journal of Bacteriology, 198(19): 2572-2578.
19. Luo, Y.; McLean, D. T.; Linden, G. J.; McAuley, D. F.; McMullan, R. and Lundy, F. T. (2017). The naturally occurring host defense peptide, LL-37, and its truncated mimetics KE-18 and KR-12 have selected biocidal and antibiofilm activities against Candida albicans, Staphylococcus aureus, and Escherichia coli in vitro. Frontiers in Microbiology, 8: 544-555.
20. De La Fuente-Núñez, C.; Korolik, V.; Bains, M.; Nguyen, U.; Breidenstein, E. B.; Horsman, S., *et al*. (2012). Inhibition of bacterial biofilm formation and swarming motility by a small synthetic cationic peptide. Antimicrobial Agents and Chemotherapy, 56(5): 2696-2704.
21. Duplantier, A. J. and van Hoek, M. L. (2013). The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds. Frontiers in immunology, 4: 143.
22. Al-Sabagh, F. S. and Ghaima, K. K. (2022). Synergistic Effect of Antimicrobial Peptide LL-37 and Ciprofloxacin against Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Burn Infections. Iraqi Journal of Biotechnology, 21(2): 32-38.