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RESEARCH ARTICLE

Killing efficacy and anti-biofilm activity of synthetic human cationic antimicrobial peptide cathelicidin hCAP-18/LL37 against urinary tract pathogens

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ABSTRACT

Objectives: Cathelicidin LL37 represents one of the chemical defence components of bladder epithelial cells that include antimicrobial peptides, which also shown to have an important role in the mucosal immunity of the urinary tract by preventing adhesion of bacteria. This study aimed to determine the killing efficacy of LL37 compared to anti-biofilm activity against *Staphylococcus aureus* and *Escherichia coli*.

Methods: The 96-flat well microtiter plates were used for evaluation of killing rate by estimation of MIC-value to the clinical isolates of *E. coli* and *S. aureus* collected from patients with urinary tract infection. *S. aureus* ATCC 25923 and *E.coli* ATCC 25922 were investigated in this study. Biofilm formation on polystyrene surface was conducted by growing bacterial isolates on 96-flat well microtiter plates, stained with crystal violet. The bound bacteria were quantified by addition of ethanol 70% and measurement of the dissolved crystal violet absorbance at (OD_{630} nm) using ELISA reader.

Results: LL37 showed minimal inhibitory concentration (MIC) of 32 µg/ml against *S. aureus* and *E. coli*. The sub-MIC of LL37 was also able to eliminate about 31% and 34% of both *S. aureus* and *E. coli*, respectively. Anti-biofilm activity of LL37 showed biofilm inhibition at 1 µg/ml (1/32 MIC) to 16 µg/ml (1/2 MIC), which exhibited significant difference ($p<0.001$) against *E. coli*, whereas LL37 beyond 1 µg/ml showed significant inhibition ($p<0.001$) of biofilm against *S. aureus*.

Conclusion: The cathelicidin LL37 can be used as a broad-spectrum anti-biofilm agent rather than killing agent. *J Microbiol Infect Dis 2015;5(1): 15-20*

Key words: Cathelicidin LL37, MIC, biofilm, anti-adhesive, killing rate

Sentetik insan katyonik antimikrobiyal peptidi olan hCAP-18/LL37'nin idrar yolu patojenlerine karşı anti-biyofim aktivitesi ve öldürücü etkinliği

ÖZET

Amaç: Cathelicidin LL37 mesane epitel hücrelerinin kimyasal bir savunma komponenti olup antimikrobiyal peptitler arasında idrar yolunda bakterilerin yapışmasını engelleyerek mukozal bağışıklıkta önemli bir role sahiptir. Bu çalışmada LL37'nin öldürücü aktivitesini *Staphylococcus aureus* ve *Escherichia coli*'ye karşı oluşan anti-biyofilm aktivitesi ile karşılaştırmayı amaçladık.

Yöntemler: İdrar yolu enfeksiyonu olan hastalardan toplanan *E. coli* ve *S. aureus* klinik izolatlarının öldürücü oranlarının tahmini MIC değerinin değerlendirilmesi için 96'lık düz kuyucuklu mikropleytler kullanıldı. Bu çalışmada, *S. aureus* ATCC 25923 ve *E. coli* ATCC 25922 suşları incelenmiştir. Polistiren yüzey üzerinde biyofilm oluşumu kristal mor ile boyalı 96'lık düz kuyucuklu mikropleytlerde üreyen bakteri izolatı ile gerçekleştirilmiştir. Bağlı bakterinin, % 70'lik etanol ve çözünmüş kristal morunun ilavesinden sonra ELISA okuyucusu kullanılarak (OD_{630} nm) absorbansı ölçüldü.

Bulgular: *S. aureus* ve *E. coli*'ye karşı LL37, minimum inhibitör konsantrasyonu (MİK) 32 µg/ml'de gösterildi. LL37'nin alt MİK değerleri ile sırasıyla *S. aureus* ve *E. coli*'nin % 31 ve % 34'nü ortadan kaldırmak mümkün oldu. LL37'nin anti-biyofilm aktivitesi 1 µg/ml (1/32 MİK)'dan 16 µg/ml (1/2 MİK)'da biyofilm inhibisyonu gösterdi ve *E. coli*'ye karşı da belirgin bir fark ($p <0.001$) ortaya koydu. LL37'nin ise 1 µg/ml'nin üzerinde S aureus'a karşı biyofilmi önemli ölçüde inhibe ettiği bulundu ($p <0.001$).

Sonuç: Cathelicidin LL37 geniş spektrumlu anti-biyofilm ajan olarak öldürücü maddeler yerine kullanılabilir.

Anahtar kelimeler: Cathelicidin LL37, MİK, biyofilm, adezyon önleyici, öldürücü oran

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INTRODUCTION

The urinary tract system, except urethra is free of all microbial types. Many factors that involve in the sterility of urine are mechanical, such as emptying the urinary bladder in a regular manner. Chemical defence of bladder epithelial cell is considered to be a second line, by producing antimicrobial peptides (AMP), which recently showed to play a significant role in the first line of innate mucosal immunity.[1]

There are two groups of AMPs in mammals, these are defensine and cathelicidin. hCAP18/LL37 is the only type of the cathelicidin found in human, which first described in 1995.[2] The terminology of hCAP18/LL37 is referred to the human cationic antimicrobial peptide with molecular weight 18 kDa.[3] The peptide is identified as LL37 because the structure is start with two amino acid of leucines in a 37 sequences.[2]

The level of LL-37 in cells and tissues are varies, and is frequently changes in the infection sites, majority in leukocytes and epithelial tissues and in different body fluids such as urine, plasma, saliva, sweat, wounds, testis and gingival.[3] Studies demonstrated the LL37 level in neutrophils is only 0.627 µg/10^6 cells, which is barely excreted in urine, on the other hand the LL37 concentration in plasma found to be 1.18µg/ml, while in the airway fluid it is ranged between (2-5 µg/ml) in adults and neonates, respectively.[4] The LL37 levels were higher in the presence of infection, which estimated (0.2-5.9 ng/ml) in healthy children's urine, but these concentrations considerably increased to the level 312.5 ng/ml in children suffering pyelonephritis and cystitis.[4] In fact, many factors like cytokines, bacterial products and growth factors can involve increasing the level of LL37, but still the regulating mechanisms of LL37 production is not completely understood.[2]

Bacterial killing by LL37 is very rapid; this is due to the mechanism that involves intercolation and assembly of the peptides with a positive charge, which draws them electrostatically to the negative charge of bacterial membrane, leading to formation of an ion channel and further disruption of membrane integrity.[5,6] Inhibition of bacterial cell wall and protein synthesis can also be an mechanism of AMPs actions.[7] It has also been shown that AMPs is capable of binding and neutralizing lipopolysaccharides.[8] On the other hand, cathelicidin LL37 could prevent bacterial adhesion on the epithelial cell lining the urinary tract system.[9] It was found that secretion of the LL37/hCAP-18 into urine increased rapidly after bacterial contact with urinary epithelial cells.[1] To our knowledge, there are only few studies in the literature on peptides with anti-biofilm activity against urinary tract pathogens. Thus, the study aimed to evaluate the killing rate of LL37 in comparison to anti-biofilm action against urinary tract pathogens such as *S. aureus* and *E. coli*.

METHODS

Human cathelicidin (hCAP/LL37): (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), was chemically synthesized (purchased from Agrisera-Sweden).

Microorganisms

Clinical isolates of *S. aureus* (n=20) and *E. coli* (n=20) isolated from patients with urinary tract infection. Standard collections of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were investigated as wild type susceptible strains in this study. The study approved by the ethics committee of College of Pharmacy, Hawler Medical University, Erbil, Iraq.

Inoculum preparation

Overnight culture plates (i.e. incubated for 18 hours) from all bacterial isolates were prepared. Individual pure colonies from each isolated plate were transferred to 5 ml sterile suspension media.

Susceptibility testing

In a 96-flat well plate, 10 µl of bacterial inoculums of 1x10^5 CFU/ml (adjusted with McFarland standard 0.5) each were incubated in 200 µl of the two-fold microdilution of peptide (i.e. 0.5, 1, 2, 4, 8, 16 and 32 µg/ml) according to the recommendation of CLSI. Positive control wells contained bacteria with no peptide. After incubation at 37 Cº for 24 hours, the optical density (OD_{480} nm) of the wells was detected to quantify the bacterial killing at each peptide concentration, using ELISA reader. The killing rate was estimated for each concentration of LL37 using the following formula as described by Noore.[10]

Percentage of killing = (Control OD_{480} nm –Test OD_{480} nm) / Control OD_{480} nm) x 100

Biofilm assay

Biofilm formation was measured with the following modifications. The 1 x 10^5 CFU/ml bacteria in 200 µL of sterile centrifuged urine was incubated with different concentrations of peptide as described previously.[11] The positive control was bacteria in

sterile urine with no peptide. To avoid cross-contamination, each bacterial isolate was allocated to one microtiter plate. Each sample was repeated for five times. After incubation at 37 ºC for 24 hours, unbound bacterial cells were removed from all wells by washing with PBS pH 7.[2], using ELISA washer for three times, then the wells exposed to air-dry and stained with 200µl crystal violet of 0.1%. Following incubation for 30 min at room temperature, the wells washed off using distilled water and set aside for air-dry. Quantification of bound bacteria performed by addition of 200 µl ethanol 70%, while dissolved crystal violet was measured at (OD_{630} nm) using microtiter plate ELISA reader. The biofilm degree was estimated based on the absorbance values obtained for individual isolates as described by.[12]

The inhibition percentage of biofilm was calculated by the formula

Percentage of biofilm inhibition = (Control OD_{630} nm –Test OD_{630} nm) / Control OD_{630} nm) x 100

Statistical analysis of data

The mean ±SD of biofilm formation were measured and the paired sample t-test was applied for comparison the means.

RESULTS

Antibacterial activity of LL37

The antibacterial potency of LL37 increased proportionally with concentration such that sub-MIC 16 µg/ml (i.e 1/2 MIC) caused a significant killing rate in *E.coli* isolates (P<0.001), while all other sub-MIC values did not (P>0.05) as shown in (Table 1). Moreover, sub-MIC 16 µg/ml level of LL37 was able to eliminate approximately 31% and 34% of growth in comparison with control growth of both *S. aureus* and *E. coli*, respectively (Figure 1).

The results confirmed by the experiments performed with the ATCC strains, which showed a maximum killing rate against 29% *S. aureus* ATCC 25923 and 35% for *E. coli* ATCC 25922 (Figure 1).

Table 1. Mean of bacterial growth by six sub-MIC levels of LL37 against *S. aureus* and *E. coli*

LL37 µg/ml	Mean Growth ($OD_{480\ nm}$)			
	***E.coli* (n=20)**		***S.aureus* (n=20)**	
	Mean ± SD	**p-value***	**Mean ± SD**	**p-value***
Control	0.589 ±0.088		0.581 ±0.151	
0.5	0.586 ±0.086	> 0.05	0.575 ±0.159	> 0.05
1	0.538 ± 0.096	> 0.05	0.542 ±0.156	> 0.05
2	0.514 ±0.107	> 0.05	0.501 ±0.146	> 0.05
4	0.478 ±0.111	> 0.05	0.467 ±0.143	> 0.05
8	0.44± 0.116	> 0.05	0.424 ±0.139	> 0.05
16	0.386 ±0.106	<0.001	0.403 ±0.150	> 0.05

* P-value represents the comparison between control and different concentrations

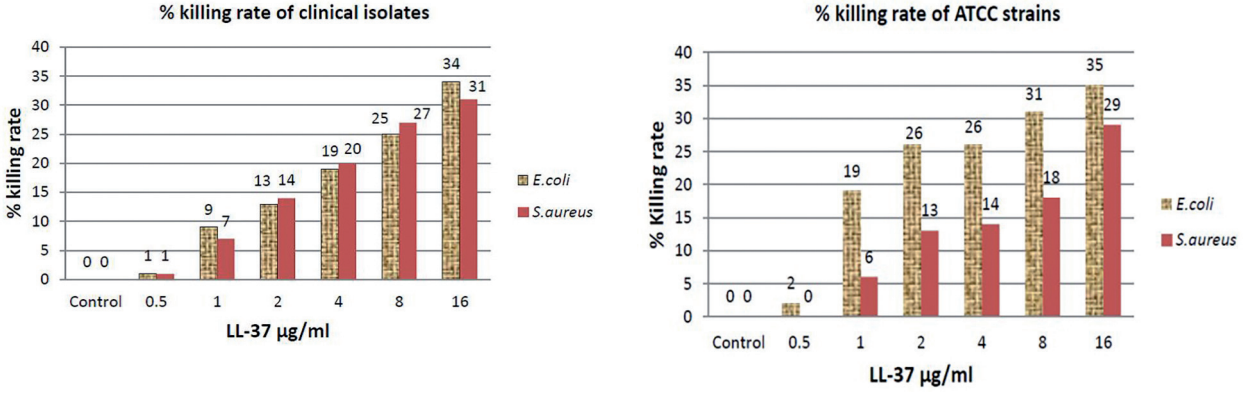

Figure 1. Killing rate of bacterial growth by six sub-MIC levels of LL37 against clinical and ATCC strains of *S. aureus* and *E. coli*

Anti-biofilm activity of LL37

Anti-biofilm activity of LL37 at sub-MIC values showed biofilm inhibition at 1 µg/ml (i.e. 1/32 MIC) to 16 µg/ml (i.e. 1/2 MIC) range. The results exhibited significantly difference (P<0.001) against *E. coli*, whereas LL37 beyond 1 µg/ml showed significant inhibition (P<0.001) of biofilm against *S. aureus* (Table 2).

Table 2. Mean of biofilm inhibition by six sub-MIC levels of LL37 against *S. aureus* and *E. coli*

LL37 µg/ml	Mean Biofilm inhibition ($OD_{630\ nm}$)			
	***E.coli* (n=20)**		***S.aureus* (n=20)**	
	Mean ± SD	**p-value***	**Mean ± SD**	**p-value***
Control	0.291 ± 0.164		0.151 ±0.032	
0.5	0.246 + 0.118	> 0.05	0.147 ±0.034	> 0.05
1	0.188 + 0.042	< 0.05	0.131 ±0.030	> 0.05
2	0.131 ±0.017	<0.001	0.101 ± 0.011	<0.001
4	0.112 ±0.072	<0.001	0.094 ±0.014	<0.001
8	0.1 ±0.027	<0.001	0.084 ±0.011	<0.001
16	0.093 ± 0.020	<0.001	0.081 ±0.012	<0.001

* P-value represents the comparison between control and different concentrations

Once more, these results confirmed by standard strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922as shown in (Figure 2).

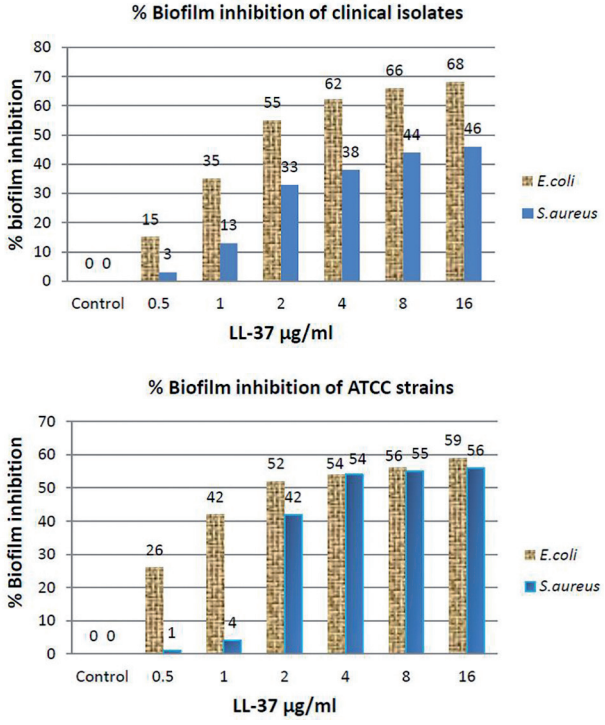

Figure 2. Percentage biofilm inhibition by six sub-MIC levels of LL37 against clinical and ATCC strains of *S. aureus* and *E. coli* and

Another set of the study investigated a comparison between killing rate and anti-biofilm activity of LL37 against *E. coli* and *S. aureus*. A significant difference between anti-biofilm activity (P<0.001) than killing rate of LL37 against both *S. aureus* and *E. coli* and was observed (Figure 3).

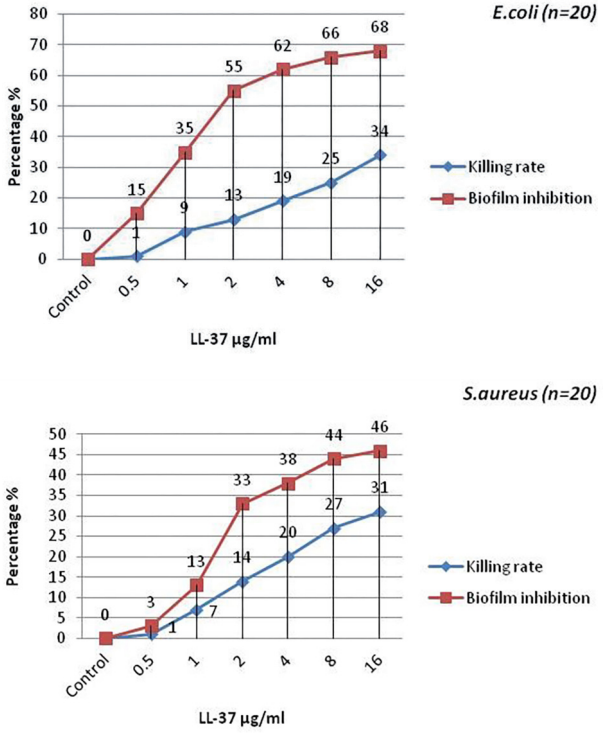

Figure 3. Percentage of biofilm inhibition and killing rate of LL37 against 20 isolates of *S. aureus* and *E. coli* and

When the effect of LL37 compared between ATCC strains and clinical isolates, *E. coli* ATCC 25922 showed more sensitivity to killing but less sensitive to biofilm inhibition than clinical isolates. In contrast, LL37 was unexpectedly more efficient in killing the clinical isolates of *S. aureus* than ATCC strains (Figure 4).

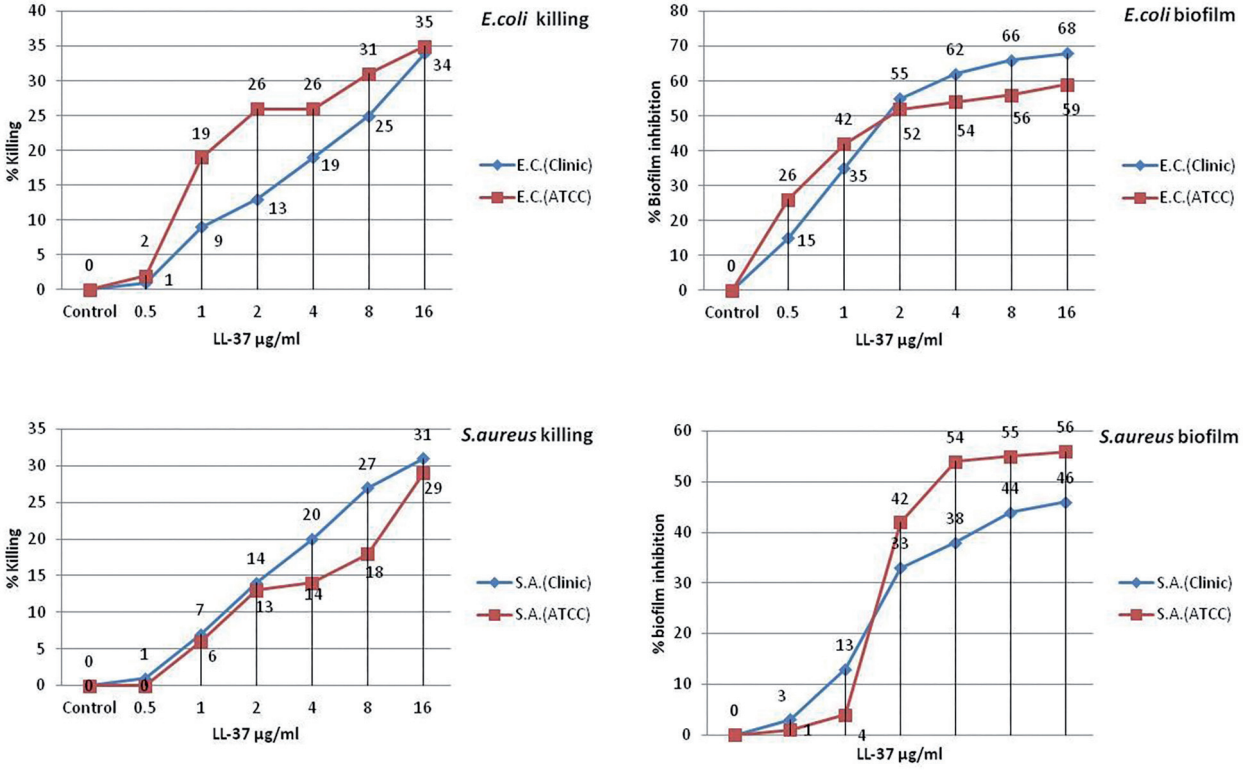

Figure 4. Percentage of bacterial killing and biofilm inhibition among clinical isolates and ATCC strains of *S. aureus* and *E. coli*

DISCUSSION

This study designed to demonstrate the role of cathelicidin LL37 as an anti-bacterial and anti-biofilm agent against clinical isolates from urine cultures of patient suffering from urinary tract infection. The study included the in vitro sensitivity of isolates from *S. aureus* and *E. coli* to synthetic antimicrobial peptide of human cathelicidin LL37. The results show a similar antibacterial effectiveness of LL37against both *S. aureus* and *E. coli*, since they displayed same *in vitro* MIC-values. In order to show the exact effectiveness of LL37 against Gram negative and positive bacteria, we convert the OD-values to percent ratios to reflect the killing percentage and biofilm inhibition. Therefore, on the percentage basis, LL37 was slightly more effective against *E. coli* than *S. aureus* in both field of the study, which included killing rate and anti-biofilm activitiy. In fact, LL37 as cationic peptide can bind to the negative charge of bacterial outer membrane by electrostatic and hydrophobic actions, consequently demonstrating the important step of killing mechanism against Gram-negative bacteria.[13-16] Moreover, recent analysis on *E. coli* revealed that binding LL37 to the O-antigen as outer layer of LPS, which develops a quickly saturation causing rapid killing.[17]

The results revealed that LL37 is stronger in anti-biofilm than killing potency. Even though the responsible mechanism for anti-adhisive action of LL-37 is unknown, a number of these mechanisms are achievable such as avoidance of initial attachment or membrane blockage of intracellular molecules. These results agreed with studies that found LL-37 concentrations at 0.5 µg/ml could inhibit the biofilm formation of *S. aureus* was faraway that required for killing growth at 64 µg/ml.[7] Therefore, the ability of low levels LL37 to inhibit biofilm formation, can be

showing signs aspect for treatment of chronic infectious diseases.[18]

According to the mentioned above, describing these peptides as anti-biofilm could be more appropriate than antimicrobial peptides, reflecting the potential role of biofilms in infection.[19] Furthermore, the study suggests different mechanisms for killing influence and anti-biofilm potency of LL37. For example, direct physical damage the bacterial membrane could develop rapid killing, while alteration of bacterial gene expression could be mediated the anti-biofilm action.[7]

This study revealed that LL-37 can display a strain-specific activity, which is exhibited that clinical isolates was more sensitive than standard ATCC strain of *S. aureus* (Figure 4). These findings indicated that clinical isolates of *S. aureus* was unexpectedly more sensitive to LL-37 than the ATCC strain. Although the reason for these observations is unknown, it might suggest a new exposure of these isolates to this agent. The results came consistent with the findings of recent study by Noorel.[10] Finally, the results obtained in the present study and in the recent scientific literature indicate that LL37 peptides should be referred to as anti-biofilm peptides.

The study concluded that the cathelicidin LL37 can be used as broad-spectrum anti-biofilm agent rather than an antimicrobial, since their anti-biofilm properties coupled with low concentrations in comparison with killing activities.

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