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ORIGINAL ARTICLE

Cathelicidin (LL-37) As a Diagnostic Marker of Urinary Tract Infection

Mohamed G. Awadallah, Gamal A. Amer, Hala A. Tabl* and Neama L. Ismail
Medical Microbiology & Immunology Department, Faculty of Medicine, Benha University

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Corresponding Author:
Hala A. Tabl
Medical Microbiology &Immunology Department, Faculty of Medicine, Benha University
Tel.: 01006243347
tablhala@yahoo.com

ABSTRACT

Background: *Cathelicidin (LL-37) comprises one of several types of antimicrobial peptides that have a vital role in the innate defense against the urinary tract infection (UTI).* ***Objectives:*** *Is to evaluate the diagnostic role of LL-37 in UTI.* ***Methodology:*** *Urine specimens were collected from 70 patients with clinically suspected UTI and from 20 healthy controls. Culture of urine and sensitivity to antimicrobials were tested. LL-37 urinary levels were measured in all participants using an enzyme-linked immunosorbent assay (ELISA).* ***Results:*** *Based on the results of urine culture, the patient group were classified into: 50 patients with culture positive urine (proven UTI) and 20 patients with culture negative urine (suspected UTI). Urine from all control subjects were culture negative. There is significant rise in the level of LL-37 among proven UTI group in comparison with suspected UTI and control groups ($p<0.001$), while, there is non-significant increase in the LL-37 level among suspected UTI group when compared with control group. There is a significant positive correlation between LL-37 level and bacterial count among proven UTI group (rho=0.442, P=0.003). ROC curve showing excellent ability of LL-37 to differentiate between proven and suspected UTI (AUC=0.982, P=<0.001). The uropathogenic Escherichia coli was the predominant isolate, n = 22 (44%). 67% of the isolates were multidrug resistant (MDR). There is non-significant relation between LL-37 levels and type of organisms isolated from urine (p=0.54).* ***Conclusion:*** *LL-37 can be considered as a good diagnostic marker for UTI.*

INTRODUCTION

Urinary tract infection (UTI) is a major problem of health as it is one of the commonest bacterial infections. The predominant causative organism is Escherichia coli (E. coli) [1].

It has been found that defense against urinary tract may be greatly dependent on specific soluble mediators secreted by epithelial cells and one of them are bactericidal antimicrobial peptides (AMPs) [2]. At least two families of AMPs are known; cathelicidin and defensins. Only one type of cathelicidin is known to be found in humans, compared to the many types of defensins (alpha, beta, and gamma) [3].

Cathelicidin is found to be expressed constitutively in trace amounts in renal epithelial cells and neutrophils, in which the peptide is stored in granules [4]. Upon contact with uropathogenic organism, however, cathelicidin is synthesized and released within minutes. Such a rapid response seems to be a very important first-line defense strategy of the native immune system of the urinary tract, preceding the influx of neutrophils and occurring long before the antibodies production [5].

The product of the gene is produced as a pro-peptide and is named as human cationic antimicrobial peptide-18 (hCAP-18/LL-37). hCAP-18 is cleaved in various tissues by the effect of proteases forming two portions; the C-terminal and the N-terminal (cathelin) parts. The C-terminal part is further enzymatically cleaved to give a peptide of 37 amino acids that starts with two amino acid leucines, and this is why named LL-37. LL-37 is considered the main endogenous broad spectrum biologically active antimicrobial agent [6].

LL-37 is an amphipathic, α-helical peptide, which preferentially binds to negatively charged parts on the outer layer of the bacterial membrane thus inducing its damage. Cathelicidins can also inhibit the bacterial biofilm formation and immune-modulate many processes of adaptive and innate immunity [7].

During inflammation, it is a potent chemoattractant for immune cells and may stimulate production of chemokines and cytokines by several cell types. It has been found that LL-37 bind and neutralize the endotoxic activity of lipopolysaccharides (LPS), endotoxins released from Gram-negative bacteria upon cell death, through binding to them with high affinity. In addition, LL-37 found to block the binding of LPS with their specific receptors, suppressing apoptosis of endothelial cells induced by these LPS, and to inhibit the effects of flagellin and lipoteichoic acid on dendritic cells [8].

Diagnosis of UTI often represents a challenge in the Emergency Department where the results of urine culture are not available, and the other tests demonstrate limited sensitivity and specificity. Diagnosis of UTI in

old patients is, in particular, problematic due to asymptomatic bacteriuria, atypical presentations and less test performance, when compared to younger adults. So, it is difficult to diagnose acute UTIs rapidly and accurately in the Emergency Department [9].

One potential strategy to improve diagnostic accuracy uses the innate immune response. LL-37 and other AMPs represents a promising issue in this field [9]. This study was designed to evaluate the role of LL-37 as a diagnostic marker of UTI.

METHODOLOGY

This is a case–control study, conducted during the period from June 2018 to February 2019. The work was approved by Benha University Ethical Committee and a written consent was obtained from each participant in the study.

The study involved 70 consecutive patients with UTI symptoms who attended the Outpatient Clinic and the Inpatient of Urology Department, Benha University, and 20 apparently healthy volunteers without any previous or current history of UTIs as a control group.

Inclusion criteria: cases with symptoms of UTI which include: dysuria, frequency, urgency, lower abdominal or suprapubic pain, fever, pain in the flank, nausea or vomiting. Exclusion criteria: diabetic patients, pregnant women or subjects with anatomical or functional abnormalities of the urinary tract or subjects with underlying diseases or receiving any antibiotics within a period of 7-10 days were excluded from both cases and control groups.

Collection of samples:

Midstream urine samples were collected from all participants according to the clean-catch procedure. In the medical laboratory each urine sample was divided into two parts; the first part was stored at −80 °C until further processed for measuring LL-37 level by ELISA, and the second part was immediately processed for urine culture.

Urine culture and bacterial count:

By using a standard calibrated (1 µL and 10 µL) loop, uncentrifuged urine was inoculated on to Cysteine Lactose Electrolyte Deficient (CLED) agar, MacConkey's and Blood Agar (Oxoid, Basingstoke, UK). Plates were aerobically incubated at 35–37 °C for 24 h. Bacterial count was measured by counting the number of colony-forming units (CFU)/ml urine. Bacterial count was used to differentiate between true bacteriuria and vulval or urethral contamination, that can occur during urine collection. Significant UTI was defined as urine culture plates showing $\geq 10^5$ colony-forming units (CFU)/ml fresh uncentrifuged urine which is significantly diagnostic of UTI. Identification of the organisms was done by the standard bacteriological methods.

Antibiotic susceptibility:

It was done on Muller Hinton agar plates by disc diffusion (Kirby-Bauer) method and interpreted according to Clinical Laboratory Standards Institute recommendations (CLSI) [10]. The discs used were: nitrofurantoin (300µg), chloramphenicol (30µg), vancomycin (30 µg), ofloxacin (5 µg), asteroname (10 µg), cephradin (30 µg), ceftriaxone (30 µg), ampicillin (10 µg), gentamycin (10 µg) and ciprofloxacin (5 µg).

Assessment of urinary LL-37 level:

It was done by ELISA kit (HK321–02 Hycult Biotech,Germany).

Test principle:

It is a quantitative sandwich enzyme immunoassay. Standards and samples react with captured specific antibody coated on the microtiter well. Biotinylated antibody reacts with captured human LL-37. Streptavidin-peroxidase is added and reacts with the biotinylated antibody. Streptavidin-peroxidase reacts with tetramethylbenzidine (TMB). Oxalic acid added to stop the reaction. The absorbance is measured with a spectrophotometer at 450 nm. To obtain the standard curve, the concentrations of the standards of human LL-37 (log) plotted against the corresponding absorbance (linear). The levels of LL-37 can be detected from the standard curve.

Statistical Analysis:

The collected data were tabulated and analyzed using SPSS version 16 soft ware (SpssInc, Chicago, ILL Company. The collected data were summarized in terms of number and percentage (for qualitative data) and mean ± SD, median, IQR and range (for quantitative data). Quantitative data were tested for normality using Shapiro-Wilks test, assuming normality at $P>0.05$. Difference among 3 independent means of LL-37 was analyzed using Kruskal Wallis test (KW) as it was proved a non parametric variable. Non parametric correlations were tested using Spearman's correlation coefficient (rho). ROC curve was constructed to detect cutoff value of LL-37 with optimum sensitivity and specificity in differentiating proven from suspected UTI. $P \leq 0.05$ was considered significant.

RESULTS

Based on the results of urine culture, the 70 patients with clinically diagnosed UTI were classified into two groups:

- Culture positive (proven UTI) group (n= 50). They were 13 males (26%) and 37 females (74%) with mean age of 30.6±7.1 years.
- Culture negative (suspected UTI) group (n= 20). They were 15 females and 5 males with mean of age 31.7±7.8 years.

All subjects of control group were culture negative.

The level of LL-37 showed a highly significant increase in proven UTI group when compared with suspected UTI and control groups (p<0.001), however, there was non-significant difference between suspected UTI group and control group (p= 0.22) (Table 1).

Table 1: LL-37 levels in the studied groups.

	Proven UTI group (n=50)	**Suspected UTI group (n=20)**	**Control group (n=20)**	**KW test P**
	Mean± SD (Range)	**Mean± SD (Range)**	**Mean± SD (Range)**	
LL-37 level (ng/ml)	63.9 ± 22.1†‡ (30.7-110.6)	20.6 ± 11.7 (10.2-60.6)	10.3 ± 9.9 (0.13-26.7)	62.9 <0.001 (HS)

KW→Kruskal Wallis test. HS: Highly significant. NS: Non-significant.
†→Significant in comparison with controls
‡→ Significant in comparison with suspected group

There was non-significant correlation between LL-37 level and age of patients of proven UTI (rho=-0.067,p=0.64) and suspected UTI (rho=0.404, p=0.077). There was also non-significant relation between LL-37 level and sex of patients of proven UTI (p=0.25) and suspected UTI (p=0.09).

There was a significant positive correlation between LL-37 level and bacterial count (rho= 0.442, p= 0.003) (Figure 1).

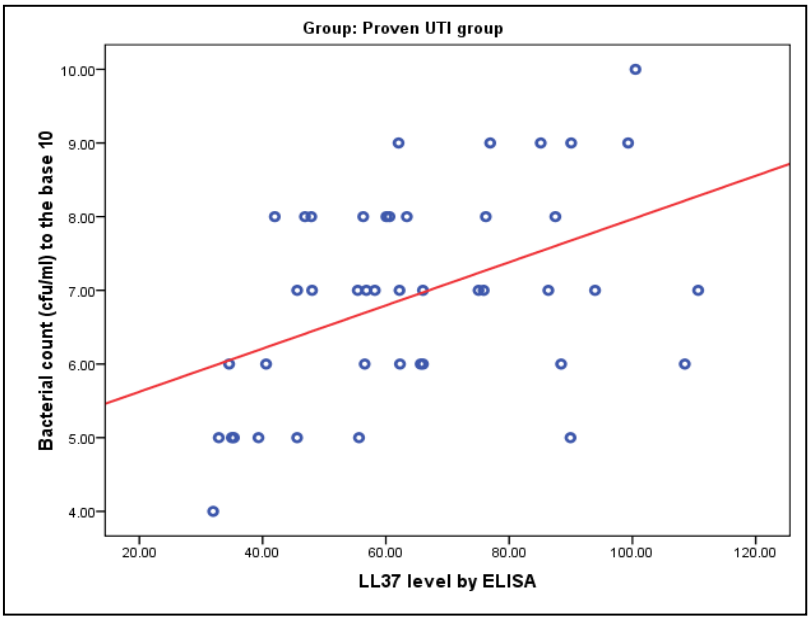

Fig. 1: Scatter graph showing significant positive correlation between LL-37 level and bacterial count using Spearman's correlation coefficient (rho= 0.442, p= 0.003).

The results of urine culture showed that *E-coli* (44%) is the most frequently isolated organism followed by *Klebsiella pneumoniae* (30%), *Coagulase-negative staphylococci* (14%), *Staphylococcus aureus (6%)* and *Pseudomonas aeruginosa* (6%). 67% of the isolates were multi-drug resistant (MDR) i.e. non-susceptibility to one agent (at least) in three or more antibiotic categories.

There was no significant relation between LL-37 level and type of isolated organism (p=0.54) (Table 2).

Table 2: Variations in LL-37 levels according to types of the organisms isolated from urine.

Organism	n.	LL-37 level (ng/ml)		KW test	P value
		Mean ± SD	Range		
E. Coli	22	60.1 ± 16.99	32.9-93.9	3.09	0.54 (NS)
Klebsiella pneumoniae	15	72.8 ± 25.90	35-110.7		
Coagulase –ve staphylococci	7	53.1 ± 10.59	45.6-60.6		
Staphylococcus aureus	3	63.7 ± 28.28	32-89.9		
Pseudomonas aueroginosa	3	60.1 ± 26.15	30.7-109.5		

KW→Kruskal Wallis test. NS: Non-significant.

At cutoff value of ≥26.6 ng/ml, ROC curve analysis revealed excellent ability of LL-37 to differentiate proven from suspected UTI. The sensitivity, specificity, PPV, NPV, Accuracy and AUC were 100%, 90%, 96.2%, 100%, 97.1% and 0.963, respectively (p <0.001) (Figure 2).

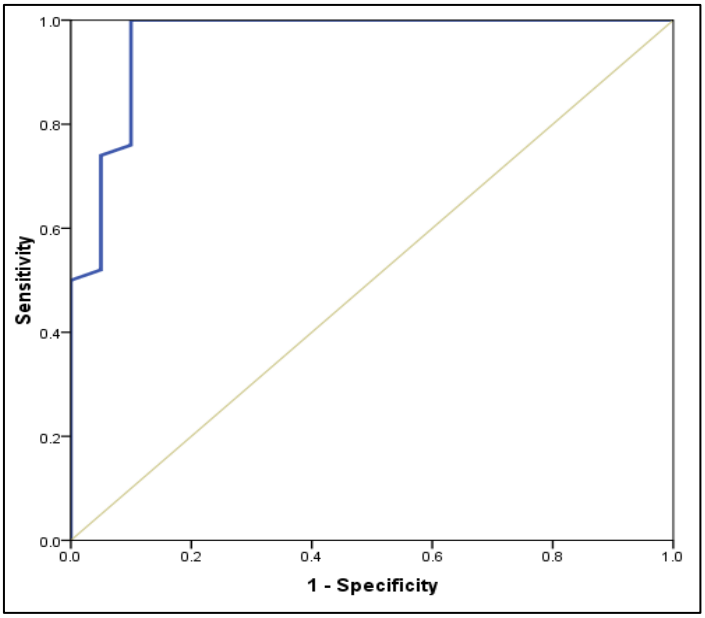

Fig. 2: ROC curve for the performance of LL-37 in differentiation between proven UTI and suspected UTI.

DISCUSSION

This work is one of few works that studied the diagnostic role of LL-37 in UTI [8,9,11,12,13]. By measuring the level of LL-37 in our participants, we found a significant increase of its level among the culture positive (proven UTI) group when compared with culture negative (suspected UTI) and control groups (p<0.001). In a study done in Sudan by *Babikir et al.*,[8] the authors assessed both the plasma and urinary LL-37 levels and they reported that its level is significantly increased in UTI patients when compared to healthy controls, which comes in accordance with our results. Also, *Chromek et al.*,[11] found significant increase in LL-37 in children with pyelonephritis or cystitis when compared with healthy children. In addition, Nielsen et al.,[12] reported that, the LL-37 urinary levels were higher during infection than post infection.

In contrast to these results, *Caterino et al.*, [9] found that LL-37 was not increased with positive cultures. Also, *Hacıhamdioğlu et al.,*[13] found that the LL-37 urinary levels in the children with UTI showed no significant differences when compared with the control group. They suggested that this non-significant difference may be related to the state of vitamin D deficiency in those patients. When they divided their patients according to their levels of vitamin D, they found that patients with sufficient vitamin D levels had higher LL-37 levels than the controls who also had sufficient levels of vitamin D. According to these

results, they suggested that sufficient vitamin D may be required to increase urine LL-37 levels during a UTI and thereby, LL-37 expression is vitamin D dependent.

Cathelicidin is expressed constitutively in the urinary tract. Direct contact with the microorganisms stimulates epithelial cells of the urinary tract to increase production of cathelicidin which protect the urinary tract from microbial adherence, and the level of production correlates with heaviness of infection [5]. This finding is in agreement with the result of the current study as we found that the urinary level of LL-37 positively correlated with the bacterial count.

Regarding sex of the patients, the present study found non-significant difference in the level of LL-37 between males and females. In agreement, *Babikir et al.*,[8] found non-significant difference in LL-37 urinary levels between males and females, however, plasma LL-37 showed a considerable increase among the females.

The current study reported that, *E-coli* (44%) is the commonest organism isolated from the urine, followed by *klebsiella pneumoniae* (30%). This result comes in agreement with several works that all found that *E.coli* is the commonest organism isolated in UTI patients [14,15,16,17].

The present study showed non-significant relation between LL-37 level and type of the isolated organisms. This result comes in agreement with another study [8] that reported that there is no significant difference in both plasma and urinary LL-37 levels when comparing isolates of *E. coli* and other uropathogens.

The diagnostic performance of LL-37 was assessed in this study, ROC curve was constructed and concluded the ability of LL-37 to differentiate proven UTI from suspected UTI (AUC=0.982). This conclusion agreed with that by another study [8] which reported that measuring levels of LL-37 could save time in diagnosing and differentiating cases with UTI from suspected UTI and so, limiting unnecessary administration of antibiotics for suspected urinary infections.

In the current study, we tested the isolated organisms against different groups of antibiotics and the result showed that 67% of the isolates were MDR. Increasing antimicrobial overuse and subsequent emergence of resistant and multi-drug resistant microbes, stimulated the attention and interest concerning the endogenous defense. Enhancement of natural endogenous defense will be an interesting new therapeutic line in the treatment of UTIs [8]. LL-37 had have potent antimicrobial activity than synthetically used antimicrobial agents. Interestingly, LL-37 directly targets the lipid membrane of bacteria, interfering with the structure of the membrane, making it different from how conventional antibiotics act by interfering with protein components of the membrane. Proteins can frequently affected by mutation, leading to antibiotic resistance, however, lipids are not affected and thus the possibility of resistance to LL-37 are very low [18]. According to these data, this work recommends more researches in this field and more attention to this group of AMPs as a promising category of antimicrobial agents.

CONCLUSION

Measuring the level of LL-37 in clinically suspected cases of UTI, can accurately make an early decision about whether there is an infection or not, and so, LL-37 could act as a good marker for diagnosing UTIs.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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