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*Original Research Article*

# Antimicrobial peptide LL 37 as a novel marker for diagnosis of early onset neonatal sepsis

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**Abstract**

**Neonatal sepsis (NS) is a potentially life-threatening clinical condition that requires early intervention. Antimicrobial peptides represent the first line of defense against invading pathogens. In addition to its broad spectrum of bactericidal activities, cathelicidin (LL-37) has a wide range of inflammatory/immune modulatory actions. The aim of this work is to assess whether the serum levels of cathelicidin (LL-37) is a useful marker in the diagnosis of neonatal sepsis, and detect its role in establishing the diagnosis and evaluating the prognosis of neonatal sepsis. The study included 30 full term newborn with clinical sepsis within the first 48 h of life. They were selected from the NICU of Al–Zahraa hospital, Al-Azhar University during the period from March 2014 to September 2014. Also the study includes 30 healthy newborn age and sex matched as a control group. Complete blood picture (CBC), differential leukocyte count, blood culture, hsCRP as well as serum level of LL-37 and IL-1β were assessed in both groups. In this study, we found a significant increase in the immature WBCs and decreases in platelets counts in the newborn with clinical sepsis than the control group. The mean serum levels of hsCRP, LL-37 and IL-1β were significantly higher in the newborn with clinical sepsis than the control group. The cut-off value of LL-37 was >0.31 ng/ml, the test was found to have a sensitivity of 93%, specificity of 93%, positive predictive value of 93%, negative predictive value of 93%. The cut-off value of IL-1β was >0.5 Pg/ml. with 86.67% and 100% sensitivity and specificity respectively, the cut of value of hsCRP and I/T were >0.41ng/ml and >0.076 respectively with 93%, 93.33% and 53%, 60% sensitivity and specificity respectively. There is a negative relation between IL-1β and the neonatal survival, while there is no significant relation between LL-37 and the neonatal survival in spite the observed decrease in its level in the neonates' non-survivors. Antimicrobial peptide LL-37 is a novel marker of early diagnosis of neonatal sepsis and can be used in combination with other acute-phase reactants to follow neonates with early sepsis.**

**Keywords:** Neonatal sepsis, LL-37, IL-1β, I/T ratio

## INTRODUCTION

Neonatal sepsis is a complex clinical syndrome that results from a systemic inflammatory response to infection in the first month of life (Pal *et al., 2014*). Early diagnosis of neonatal sepsis is challenging because clinical characteristics are nonspecific and difficult to differentiate from those of noninfectious etiologies, and the accurate laboratory tests are limited and not always reliable (Arif *et al., 2013*).

Blood culture remains the gold standard for diagnosis of neonatal sepsis, but its rate of positivity is low *(Gonzalez et al., 2013)*. Therefore, predictive markers to identify high risk patients are urgently needed for early

detection and treatment of sepsis *(Zhang, 2014)*.

During sepsis, a cascade of events is initiated by microorganisms and their derived products, with an exacerbated production of both pro and anti-inflammatory cytokines. Interleukin-1β (IL-1β) is a critical mediator of immune response to sepsis *(Netea, 2010)*. Excessive IL-1β production is directly linked to the development of shock, multi-organ system failure, systemic inflammatory response syndrome, and septic shock *(Barksby et al, 2007; Zhang, 2014)*.

Cathelicidins are cationic amphipathic peptides that span a functionally diverse repertoire to enable homeostasis in response to pathogen, disease and danger-associated molecular patterns *(Matzinger, 2002; Zanetti, 2005)*. Cathelicidins are produced by leukocytes and epithelial cells and display anti-microbial activity against a broad spectrum of bacteria, fungi and enveloped viruses *(Pazgier et al., 2013)*. The only human member of the cathelicidin family identified is LL-37, the C-terminal part of the human cationic antimicrobial protein 18 (hCAP-18) *(Kahlenberg, 2013)*.

The immunomodulatory properties of LL-37 have been widely studied in highly controlled in vitro systems *(Barbeiro al., 2013)*. However, the effects of LL-37 in the context of a highly complex immune response in the presence of other immune mediators are only beginning to be explored *(Bowdish et al., 2004; Chakraborty et al.,2008)*.

We aimed to assess whether the serum level of LL-37 is a useful marker in the diagnosis of neonatal sepsis and also disclose its relationship with cases of neonatal survival.

## Patients and methods

This is a prospective comparative study; it was carried out in the NICU of AL-Zahraa hospital, Al-Azhar University Cairo, Egypt during the period from March 2014 to September 2014. 60 full term neonates were enrolled in the study, 30 with clinical sepsis in their first 48 hours of life diagnosed according the criteria of sepsis. The clinical and historical features used to identify patients at risk for sepsis included one or more of the following, as determined by the attending neonatologist *(Gonzalez et al., 2003; Klinger et al.,2009; Benitz et al.,1999)*. (1) respiratory compromise (e.g. tachypnea, increase in frequency or severity of apnea, need for ventilator support); (2) cardiovascular compromise (e.g. increase frequency or severity of bradycardia episodes, pallor, decreased perfusion, hypotension); (3) metabolic changes (e.g. temperature instability, feeding intolerance, glucose instability, metabolic acidosis); (4) neurologic changes (e.g. lethargy, hypotonia, irritability); and (5) antenatal risk factors (e.g. maternal GBS colonization without adequate intrapartum prophylaxis, unknown maternal GBS status, maternal fever, chorioamnionitis, preterm labor, and prolonged rupture of membranes).

The case group included 22 males and 8 females, in addition to 30 age and sex matched newborn served as a control group. Informed consent was obtained from the participating parents in adherence with the guidelines of the ethical committee of AL-Zahraa hospital, AL-Azhar University, Cairo, Egypt. Preterm infants, newborns with congenital malformations, chromosomal abnormalities, suspected inborn error of metabolism and infants with perinatal asphyxia were excluded from the study. All studied neonates were subjected to: 1-Full history taking including: -Prenatal history including maternal conditions during pregnancy (diseases or medications). Natal history including mode and duration of delivery, premature rupture of membrane). -Post natal history including 1st cry, cyanosis, sleepiness, respiratory distress, convulsion, APGAR score determination at one minute, 5 and 10 minutes. 2-Clinical examination: Full general and local examination including gestational age assessment using New Ballard Score *(Ballard and Khoury, 1991)*. 3-Laboratory investigations: including CBC and differential leukocyte count, blood culture hsCRP, cathelicidin (LL-37) and IL-1β.

### Blood sampling

Five ml of venous blood samples were drawn; the samples were divided into two portions:

- Two ml were placed into a vacutainer tube containing EDTA for complete blood picture (CBC) and differential leukocytes count using automated cell counter model Sysmex KxN21.
- Three ml were placed into a plain tube, centrifuged within 30 minutes of collection and serum was separated and stored at -20° c for cathelicidin, hsCRP and IL-1β until time of assay.
- Another two ml of venous blood were drawn under complete aseptic condition for blood culture using fully automated Bact/Alert 3D 60 .
- Assessment of cathelicidin (LL-37) ng/ml, IL-1β Pg/ml and hsCRP ng/ml in the serum of all the newborn enrolled in the study using enzyme-linked immunosorbent assay (ELISA), using a SLT spectra ELISA reader (SLT lab instruments, Salzburg, Austria).
- The kit for hsCRP was supplied by immunospec, (catalog no.E29-056).
- The kit for IL-1β was supplied by assaypro (catalog no.E12200-1).

### Cathelicidin test procedure summary

Cathelicidin was detected by ELISA immunoassay kit from Glory science .

**Table 1.** Demographic data, anthropometric measurements, perinatal events and laboratory data of the newborn with clinical sepsis and the controls

| Variable | Cases group (n = 30) (Mean ± SD) | Control group (n = 30) (Mean ± SD) | Statistical test $t/X^2$ | P-value |
|---|---|---|---|---|
| **Gestational age** (wks) | 38.13 ± 1.17 | 38.13 ± 1.17 | 0.000 | 1.000 |
| **Sex** | | | | |
| Male | 22 (73.3%) | 18 (60.0%) | 1.200 | 0.273 |
| Female | 8 (26.7%) | 12 (40.0%) | | |
| **Birth weight** (gm) | 3220 ± 617.22 | 3160 ± 376.55 | 0.455 | 0.651 |
| **Length** (cm) | 47.93 ± 3.74 | 47.67 ± 2.56 | 0.322 | 0.749 |
| **PROM** | 10 (33.33%) | 0 (0%) | | |
| **Mode of delivery** | | | | |
| Vaginal delivery | 12 (40%) | 12 (40.0%) | 0.000 | 1.000 |
| Cesarean section | 18 (60%) | 18 (60%) | | |
| **APGAR score** | | | | |
| 1min | 7.13±0.97 | 7.53±0.51 | - 1.996 | 0.051 |
| 5min | 8.67±0.48 | 8.73±0.45 | - 0.555 | 0.581 |
| **CBC with differential** | | | | |
| Hb (g/dL) | 14.91±2.25 | 16.03±2.85 | -2.287 | 0.026 ** |
| TLC ($\times 10^9$/L) | 13.81±5.15 | 14.03±1.4 | - 0.211 | 0.834 |
| I/T ratio | 0.18±0.09 | 0.08±0.03 | 5.971 | 0.000 ** |
| Platelets ($\times 10^9$ /L) | 265.2±80.75 | 310.93±40.71 | -2.77 | 0.008 ** |
| **Serum biomarkers** | | | | |
| hsCRP (ng/dL) | 1.05 ± 0.79 | 0.52 ± 0.36 | 3.355 | 0.001** |
| IL-1β (Pg/ml) | 1.57±0.74 | 0.34 ± 0.13 | 8.948 | 0.000** |
| Cathelicidin LL-37 ( ng/ml) | 1.91 ± 0.93 | 0.19 ± 0.08 | 10.066 | 0.000** |
| **Positive blood culture** | 12 (40%) | 0 (0%) | | |

The kit uses a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of human (cathelicidin LL-37) in samples. Add (cathelicidin LL-37) to monoclonal antibody Enzyme well which is pre-coated with human (cathelicidin) monoclonal antibody, incubation; then, add (cathelicidin LL-37) antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme.

Then add chromogenic solution A, B, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the human substance (cathelicidin LL-37) of sample were positively correlated. The color changes were measured spectrophotometrically at a wave length 450 nm. The concentration of cathelicidin (LL-37) in the sample was determined by comparing the optical density of the samples to the standard curve. The results were expressed as ng/ml. Detectable limit of cathelicidin (LL-37) ranged from 0.5 ng/ml -200 ng/ml and sensitivity 2.68 ng/ml *(Gambichler et al., 2011)*.

### Statistical Analysis

The data were revised, coded, and entered to the PC computer and analyzed using *SPSS* (version 16).

### Spearman correlation

Spearman correlation was used to assess the relation between two parameters. The P-value was considered significant as the following:

- P> 0.05: • Non significant
- P < 0.05: *Significant
- p < 0.01: **Highly statistically significant

## RESULTS

Table 1 shows significant decrease in Hb level and platelets counts in cases compared to the controls, while there is significant increase in the immature WBCs in cases than in controls, as the I/T ratio in cases and the controls were 0.18 ± 0.090, 0.08 ± 0.03 respectively with ($p < 0.01$). Also it shows significant increase in the serum level of hsCRP, IL-1β and cathelicidin (LL-37) in the newborn with clinical sepsis compared to the controls. The same table shows that blood culture was positive in 12 (40%) out of 30 newborn with clinical sepsis.

Table 2 shows significant increase in the serum level of IL-1β in the newborn with proven sepsis than in culture negative group, while there is no significant difference between both groups regarding hsCRP and cathelicidin (LL-37)serum levels.

**Table 2.** Comparison between serum level of hsCRP, IL-1β and cathelicidin (LL-37) in culture positive and negative groups

| Variable | Culture positive (n=12) (Mean ± SD) | | | Culture negative (n=18) (Mean ± SD) | | | t | p-value |
|---|---|---|---|---|---|---|---|---|
| hsCRP ng/dL | 1.3665 | ± | 1.02 | 0.84 | ± | 0.52 | 1.659 | 0.118 |
| IL-1β Pg/ml | 1.8873 | ± | 0.53 | 1.36 | ± | 0.80 | 2.159 | 0.040* |
| LL-37 ng/ml | 1.923 | ± | 0.72 | 1.90 | ± | 1.07 | 0.067 | 0.067 |

**Table 3.** Comparison between serum levels of hsCRP, IL-1β and cathelicidin (LL-37) in the newborn who survived and who are not survivor

| Variable | Not survivor (n=14) | Survivor (n=16) | t | P-value |
|---|---|---|---|---|
| hsCRP ng/L | 1.1 ± 0.79 | 1.0 ± 0.81 | 2.048 | 0.728 |
| IL-1β Pg/ml | 2.02 ± 0.24 | 1.18 ± 0.82 | 3.683 | 0.001* |
| (LL-37) ng/ml | 1.62 ± 0.98 | 2.16 ± 0.83 | -1.629 | 0.115 |

**Table 4.** Receiver operating characteristic curve (ROC) of I/T ratio, hsCRP, IL-1β and LL-37 for diagnosis of early neonatal sepsis

| Variable | Cut off point | AUC | Sensitivity | Specificity | +PV | -PV |
|---|---|---|---|---|---|---|
| I/T | < 0.076 * | 0.076 | 93.33 | 60.00 | 70.0 | 90.0 |
| hsCRP ng/dL | < 0.41 | 72.2 | 93.33 | 53.33 | 66.7 | 88.9 |
| IL-1β Pg/ml | < 0.54 | 95.8 | 86.67 | 100.00 | 100.0 | 88.2 |
| LL-37 ng/ml | < 0.31 | 95.8 | 93.33 | 93.33 | 93.3 | 93.3 |

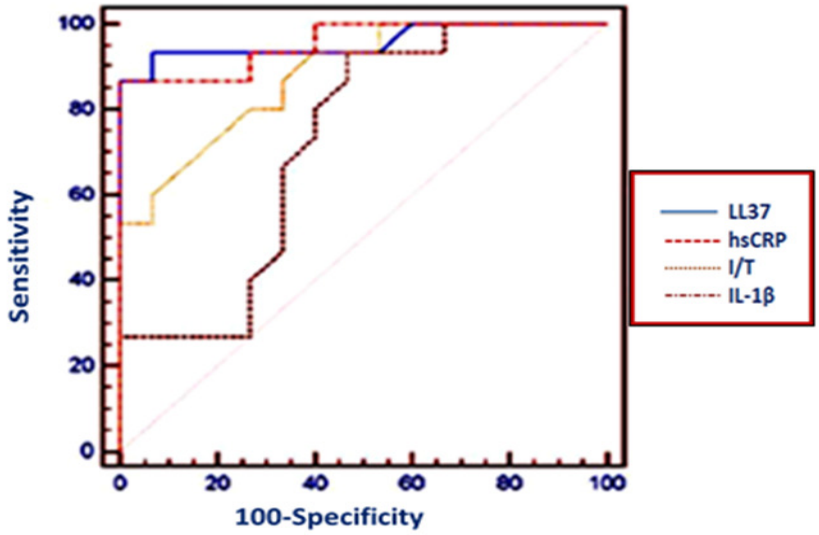


**Figure 1.** Receiver operating characteristic curves for sepsis group versus control group regarding LL-37, hsCRP, I /T ratio, and IL-1β.

Table 3 shows significant increase in the serum level of IL-1β in non-survivor newborn, on the other hand, it shows decrease in the serum level of LL-37 in the newborn who are not survivor but it was not reached to be of statistical significant value, also there is no significant difference between both groups regarding hsCRP.

Table 4 shows cut off points for I/T, hsCRP, IL-1β and LL-37; it revealed that I/T ratio 93.33% sensitive and 60% specific, hsCRP 93.33% sensitive and 53.33% specific, IL-1β 86.67% sensitive , 100.00% specific, while LL-37 93.33% sensitive, 93.33% specific.

## DISCUSSION

Accurate and quick diagnosis is essential for both protecting the infant from the consequences of the bacterial invasion and preventing damages deriving from the unnecessary use of antibiotics. The need for a supplemental test for the diagnosis of neonatal sepsis with high sensitivity and specificity is readily apparent.

The goal of this study was to investigate the value of LL37 measurements for making a diagnosis of neonatal sepsis by comparing it with I/T ratio, hsCRP and IL-1β, as it is the first study which concerned to assess cathlecidin LL37 as an early marker of neonatal sepsis. In the present study it is not surprisingly to find significant elevation in the serum level of hsCRP, IL-1β, increased I/T ratio as well as we found significant elevation in serum cathlecidin LL37 in the newborn with sepsis than in controls.

Most clinicians rely on the CBC and its derived indices, including the absolute neutrophil count, and the immature: total neutrophil ratio *(Benitz, 2010)*. Individually they do not possess high specificity or sensitivity, and are generally more helpful when considered together *(Benitz, 2010; Mishra et al., 2006; Ng and Lam, 2010; Rodwell, 1988)*.

Immature granulocytes (IGs) by microscopic count necessitate experienced laboratory staff; furthermore morphological classification of IGs is subject to a considerable reader bias and interpretative errors; especially in neonates *(Rodwell et al., 1988; Buttarello and Plebani, 2008)*. In the present study the I/T-ratio showed sensitivity 93.33% specificity 60%, positive predictive and negative predictive values 70.0% and 90% respectively so the ideal marker does not apply to I/T-ratio.

Recent development of hsCRP assay has opened up the field for wider use of CRP in diagnostics. It is one of the most widely available; most studied, and most used laboratory tests for neonatal bacterial infection. However, the study provides high sensitivity 93 % with limited specificity 53.33%). It provides very high negative predictive values and is thus useful for identifying infants unlikely to be infected or monitoring the response to treatment. Furthermore, the use of CRP in neonatal sepsis is complicated by a nonspecific rise that starts shortly after birth *(Chiesa et al., 2001; Chiesa et al., 2011)*. IL-1β is a prototypical proinflammatory cytokine, which stimulates both local and systemic inflammatory/immune responses and acts synergistically with other cytokines to cause tissue injury in sepsis, so it is not surprisingly to find high significant increase of IL-1β in the newborn who were not survivor.

An ideal marker would need to have a sensitivity of 100% with negative predictive values and PPVs of >85%, to avoid unnecessary use of antibiotics *(Luzzani et al., 2003; Tang et al., 2007; Giamarellos-Bourboulis, 2004)*.

With this study, we managed to demonstrate a specificity and PPV of 100% for LL37, similar to the other acute-phase reactants. The sensitivity and negative predictive value of LL37 were 93% and 93%, respectively, values that were higher than the most obtained for the other markers. It is not only a novel marker for neonatal sepsis diagnosis but also give an idea about the host protection status against bacterial invasion. Neutrophil-derived LL37 is central to host protection in bacterial invasion of the circulatory system, and the peptide's multifunctional properties make it an important template for the development of novel treatment solutions for sepsis *(Cirioni et al., 2008)*. LL-37 is highly expressed at barrier sites including respiratory and colonic epithelium, saliva and skin thus provides an important first line defense mechanism for the innate immune system to respond to infectious insults *(Dürr et al., 2006; Schauber and Gallo, 2008)*.

According to the present study results we showed that serum LL37 level was increased in the survivor newborn but not reached to be of statistical significant value, this may attributed to small sample size which is one of the study limitations.

## CONCLUSION

We demonstrated a novel role of the LL-37 as a marker of diagnosis of neonatal sepsis. It can be used in combination with other acute-phase reactants for follow of early onset sepsis.

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