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CORRELATION OF ENDOTOXIN AND hs-CRP LEVELS WITH BLOOD CULTURE IN SUSPECTED NEONATAL SEPSIS – A PILOT STUDY

Microbiology

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ABSTRACT

Neonatal sepsis is a major cause of morbidity and mortality worldwide, causing around 2.7 million deaths annually, 99% of which is attributed by developing countries. Gram negative bacteria are responsible for majority of neonatal sepsis. One of the greatest challenges to the medical fraternity is getting an accurate and early diagnosis of neonatal sepsis as new-borns have very subtle clinical presentations, unlike their adult counterparts and also due to the fact that a wide array of conditions mimic neonatal sepsis clinically .Because of limitations of blood culture like low sensitivity and time consuming, a wide spectrum of auxiliary tests using diagnostic markers like C-reactive protein (CRP), Pro-calcitonin (PCT) and Endotoxin assays has now emerged to aid in the presumptive as well as confirmatory diagnosis of neonatal sepsis. The present study was to evaluate the diagnostic efficacy of Endotoxin and hs-CRP as an alternative tool in early diagnosis of neonatal sepsis. 50 suspected case of neonatal sepsis studied, most of the cases were less than 5 days of age, 60% were positive and 48% were negative by blood culture. 84% were due to Gram negative bacterial sepsis and only 16% were Gram positive bacterial sepsis. The most common risk factor was Low birth weight (74%). Birth asphyxia (48%) followed by PROM (24%). The sensitivity and specificity of hs-CRP were 100% and 76% . The sensitivity and specificity of endotoxin were 84% and 68% . When Gram negative bacterial sepsis was alone considered, the sensitivity and specificity of endotoxin was significantly higher.

KEYWORDS

hs - CRP, Endotoxin, Blood culture, Neonatal sepsis

INTRODUCTION:

Neonatal sepsis is a common cause of morbidity and mortality in new-born infants. The incidence of neonatal sepsis according to National Neonatal Perinatal Database (NNPD)is 30 per 1000 live births [1].The risk factors include lack of antenatal care, unsupervised deliveries, prolonged labour, prematurity, low birth weight, and delay in recognition of danger signs in both mother and baby[2]. Since delay in diagnosis of severe bacterial infections in neonates may lead to fatal outcome, early detection and appropriate treatment of neonatal sepsis is very important , as it reduces the inadvertent use of antibiotics, including the cost of treatment and thereby prevents the emergence of drug resistant strains [3].

However, early diagnosis is difficult either due to the nonspecific clinical presentation of neonatal sepsis or because critically ill neonates often manifest systemic inflammatory response syndrome (SIRS) and no particular test is available with high diagnostic accuracy [4].Currently acute phase reactants, Pro-inflammatory cytokines, and Chemokines are being used to diagnose neonatal sepsis of which the commonly used markers include C-reactive protein (CRP), Pro-calcitonin (PCT) and Interleukin-6 (IL-6) [5].

CRP is an acute phase reactant, produced in the liver and a frequently used marker of sepsis, which has a half –life of 24 to 48 hours. But as it takes 10 to 12 hours to respond to an infection, it seems to be unreliable in certain conditions. Highly sensitive CRP (hs-CRP) is more reliable, as it measures the CRP levels (detection limit of even less than 3 mg/L) lower than that measured by the conventional CRP assays (detection limit of more than 6mg/L). This is crucial because neonates cannot produce sufficient amounts of acute phase reactants and respond to infection only with a smaller rise in CRP than adults [5,6].

Endotoxin or Lipopolysaccharide (LPS) is a membrane component of Gram negative bacteria plays a key role in the pathogenesis of sepsis. LPS somehow plays advantageous role to the host for inducing appropriate antibacterial defence mechanisms when released in small quantities within a localized tissue space. However, the sudden massive release of endotoxins as occurs in sepsis is clearly deleterious to the host, initiating the release of dysregulated and potentially lethal array of inflammatory mediators and pro-coagulant factors in the circulation. Hence, it is considered to be marker of bacterial infection as well as a target molecule of sepsis treatment [7]. Measurement of endotoxin level is an alternative diagnostic tool for early detection and appropriate selection of antibiotic treatment strategy for sepsis [8].

Blood culture is the gold standard for the diagnosis of sepsis, but it takes long duration of time to give results and pathogens are detected only in approximately 25% of cases with low sensitivity [4]. Hence, markers of sepsis like CRP and Pro-calcitonin are done nowadays as they give faster results .However, the test cost of Pro-calcitonin, an indirect marker of endotoxin is very high. There are numerous studies denoting the role of these two markers in neonatal sepsis but there are very few studies available for either endotoxin or hs-CRP. Thus, this pilot study will serve as a tool for assessing the diagnostic utility of Endotoxin and hs-CRP in comparsion with blood culture for early detection of neonatal sepsis.

MATERIALS AND METHODS:

This cross sectional hospital-based study was conducted in the Department of Microbiology, Coimbatore Medical College & Hospital over a period of 3 months (June 2018 – August 2018). A total of 50 suspected cases of Neonatal sepsis admitted in the Neonatal Intensive Care Unit (NICU), who satisfied the selection criteria mentioned below were included in this study. The demographic information of the cases like age in days, sex, birth weight, place and mode of delivery along with risk factors for sepsis was recorded. The study was conducted after obtaining clearance from the Institutional Human Ethical Committee and getting informed consent from the subjects' parent/legal guardian.

Inclusion criteria:

- Preterm and term neonates (< 28 days of age) of both sexes showing signs of both early and late onset sepsis were included in the study.
- Clinical signs and symptoms of sepsis include at least three of the following: Abdominal distension, temperature instability, dyspnoea, tachypnoea (RR >70/min), feeding intolerance, hepatosplenomegaly, lethargy or irritability, tachycardia (HR>190beats per minute), bradycardia (HR<90beats per minute).

Exclusion criteria:

- Neonates born with congenital anomalies or those neonates who have received antibiotics or undergone any surgical procedure were excluded

The cases under study were categorized into as (Group A):25 cases of proven sepsis (culture positive cases) and (Group B): 25 cases of probable sepsis (culture negative cases with at least three signs and symptoms of sepsis).

Blood Sample Collection:

Minimum amount of 3 - 4 ml of blood was collected by heel prick and venepuncture method following standard aseptic precautions. The

collected blood sample was inoculated in blood culture bottle containing 30 ml of trypticase soy broth and the remaining blood was transferred to clot activator tubes (for serum) for estimation of hs-CRP and to sterile heparinized tubes (for plasma) for estimation of endotoxin, which were centrifuged accordingly and placed in cold storage until use.

Blood culture by Manual Method:
The blood culture bottles are incubated at 37°C and examined daily for 7 days for evidence of growth, indicated by turbidity, hemolysis, gas production, discrete colonies, or a combination of these.Blind subculture after overnight incubation and on 5th (final) day post-incubation was made on sheep blood agar and MacConkey agar (MAC). During the 5 day period, bottles were examined daily and subcultures on solid media whenever there was a visible sign of growth in the bottle were made. Colonies were identified by biochemical tests and antibiogram was performed by disk diffusion method.

hs-CRP Detection by Nephelometry:
Principle: During the test CRP in the sample binds with the specific anti CRP antibody that is coated with latex particles to cause agglutination which is then detected optically by GB NEPHCHEM analyser. The change in the absorbance is proportional to the hs-CRP in the sample. The actual concentration is obtained by comparing with a calibration curve with known concentrations.

Reference value of hs-CRP < 3.0 mg/L Method: The test card is inserted (for Calibration) into the card reader slot ; Pipette out 150 µl of R1(glycine buffer solution Sodium Azide< 0.1%) into dedicated cuvette and add 5 µl of serum sample and place the cuvette in the reading chamber. After incubation ; add 60 µl of R2 (latex suspension, anti CRP antibodies glycine buffer Sodium Azide < 0.1%) using sensor pipette connected with the machine into the cuvette. Once the reaction time has been completed, the result will show in the display.

Endotoxin Detection by Gel clot assay:
Principle: Gram-negative bacterial endotoxin catalyzes the activation of a pro-enzyme into enzyme (i.e., coagulase) which hydrolyzes specific bonds within a clotting protein (coagulogen) present in LAL. Once hydrolyzed, the resultant coagulin self-associates and forms a gelatinous clot.

Method: Plasma samples were diluted 1:10 in 0.1 % Tween 80 and heated at 70 °C for 10 min to denature plasma proteins and proteases which may interfere with endotoxin detection by the limulus amebocyte lysate (LAL) gel clot assay . Samples were brought to room temperature (20–25 °C) prior to endotoxin assay. Reconstitute, dilute the Control Standard Endotoxin to a concentration of 1.0 EU/mL and also prepare serial 2 fold dilution from 1.0 EU/mL upto 0.015 EU/mL (lysate sensitivity) using LAL Reagent water. Add 0.25 ml of sample to be tested to the lysate test vial along with Negative control & Positive control supplied with the kit. Mix by tilting and gently swirl the test vial and incubate at 37 °C for 60 minutes. Carefully remove each vial and invert at 180°. Record the reaction with clot as positive and No clot as negative.

RESULTS:
Among the blood culture positive cases (n = 25) included in this study, 60 % culture positivity was observed in the age group of < 5 days, followed by 28% in the age group of 6 - 10 days and the remaining 12 % were more than 10 days old. A proportionate number of blood culture negative cases of similar age groups were also analyzed in detail. The blood culture positivity was higher in female (56%) than male (44%), whereas blood culture negativity was higher in males (52%) than females (48%) (Refer: Table 1).

Table1: Age & Gender wise distribution of cases under study

Age in days	Culture Positive (n = 25) (%)	Culture Negative (n = 25) (%)
< 5	15 (60 %)	12 (48%)
6-10	7 (28%)	10 (40%)
11 -15	2 (8%)	3 (12%)
> 16	1 (4%)	0 (0%)
Gender		
Male	11 (44%)	13 (52%)
Female	14 (56%)	12 (48%)

Regarding the place of delivery, we had 48% intramural delivery (baby born within the premises of our centre) and 52% extramural delivery (baby born outside our premises). 76% of suspected neonatal sepsis cases were delivered by normal vaginal delivery and the remaining cases being delivered by instrumental delivery (10%) and lower segment caesarean section (LSCS) (14%). Considering the birth weight, most of the cases (54%) were of low birth weight(LBW) i.e. birth weight of less than 2500 grams and 26% were normal birth weight(more than 2500gm) and 20% were of very low birth weight(VLBW) of less than 1500 gm. The risk factors of sepsis in majority of cases were found to be history of birth asphyxia (48%), followed by history of prolonged rupture of membrane (PROM) for ≥ 18 hours (24%) (Refer: Table 2)

Table 2 : Demographic features & Risk factors of cases under study

Risk Factors	n = 50	%
Place of Delivery		
Intramural	24	48
Extramural	26	52
Mode of Delivery		
Normal	38	76
Instrumentation	5	10
LSCS	7	14
Birth weight		
Normal	13	26
LBW	27	54
VLBW	10	20
Risk factor for sepsis		
H/o Birth Asphyxia	24	48
H/o PROM ≥ 18 hours	12	24
H/o Foul smelling liquor	9	18
H/o Maternal Fever	5	10

Among the blood culture positivity (n=25), 21 were Gram negative bacterial isolates and 4 were Gram positive bacterial isolates. Among the Gram negative bacterial isolates, Escherichia coli (38%), Pseudomonas aeruginosa (24%) and Klebseilla pneumoniae (24%) were the major etiological agents. (Table 3).

Table 3 : Organisms isolated in Blood culture (n=25)

Gram Negative Bacteria	(n =21) (84%)
Escherichia coli	8 (38%)
Klebsiella pneumoniae	5 (24%)
Pseudomonas aeruginosa	5 (24%)
Acinetobacterbaumanni	2 (10%)
Enterobacteraerogenes	1 (4%)
Gram Positive bacteria	**(n=4) (16%)**
Staphylococcus epidermidis	2 (10%)
Staphylococcus aureus	1 (4%)
Enterococcus faecalis	1 (4%)

All the blood culture positive cases (n=25) were also positive for hs-CRP(100%) , whereas endotoxin was detected only in 21 (84%) among them. Out of blood culture negative cases (n= 25) studied, 6 (24%) were found to be positive for hs-CRP and the remaining 19 (76%) were negative for hs-CRP. Similarly, only 8(32%) were found to be positive for endotoxin , whereas 17 (68%) were negative for endotoxin . It was also observed that the endotoxin level was higher (≥ 0.125 EU/ml) for culture positive cases (n = 21) compared to culture negative cases (n = 8) where endotoxin level in the lower limit of detectable range(≤ 0.006 EU/ml). (Refer Tables 4).

Table 4 : Correlation of hs-CRP & Endotoxin with Blood Culture

hs-CRP	Blood Culture		Total	Endotoxin	Blood Culture		Total
	Positive	Negative			Positive	Negative	
Positive	25	6	31	**Positive**	21	8	29
Negative	0	19	19	**Negative**	4	17	21
Total	25	25	50	**Total**	25	25	50

The sensitivity and specificity of hs-CRP in the diagnosis of neonatal sepsis was found to be 100% and 76% respectively. The sensitivity and specificity of endotoxin was found to be 84% and 68% respectively. These results were obtained by considering Gram positive and Gram negative bacterial sepsis cases altogether. However, when calculated with exclusion of sepsis due to Gram positive bacteria which lacks LPS, the sensitivity and specificity of endotoxin for detecting Gram

negative bacterial sepsis was 100% and 100% respectively.

Statistical analysis:
Data analysis was done using Chi square test and the results showed statistically significant difference in hs-CRP and endotoxin assays between culture positive and culture negative cases with p values <0.00001 and 0.000197 respectively (Ref :Table 5).

Table 5 : Significance of hs-CRP and endotoxin assays

	Chi square value	p value
hs-CRP	30.64	<0.00001*
Endotoxin	13.86	0.000197*

* = Highly statistically significant

DISCUSSION:

Neonatal sepsis is still responsible for over 1 million deaths globally every year , which is much higher in developing countries like India. Risk factors for the development of neonatal sepsis can be divided into maternal and neonatal/postnatal factors. Maternal factors include prematurity, low birth weight (<2500 grams), prolonged rupture of placental membranes (PROM), maternal intra-partum fever and chorioamnionitis. Postnatal factors include male gender, birth weight < 1000 grams, central venous catheters and prolonged duration of mechanical ventilation [17]. 48% of our subjects had a history of birth asphyxia and 24% had a history of PROM >18 hours which may be due to the fact that PROM increases the risk of ascending infections. Majority of the suspected neonatal sepsis cases were delivered by normal vaginal delivery (76%) which was similar to a study conducted by Bangi et al [2]. 74% of our subjects had low birth weight (<2500 grams) which was in accordance with similar previous studies [9,10,11].

Among the blood culture positive cases (n=25), 21 were affected by Gram negative bacteria, with Escherichia coli being the most common etiological agent followed by Pseudomonas aeruginosa and Klebseilla pneumonia. However, majority of the previous studies on neonatal sepsis reported Klebseilla pneumonia and Coagulase negative Staphylococci (CONS) to be the leading causative agents in developing countries [9,10,12].

We observed 6 cases (24%) were positive for hs-CRP among the blood culture negative cases (n= 25) , which could be explained when the bacterial load is too low and failed to be isolated in culture in condition like impeding sepsis , even the slight rise of CRP in response to bacterial invasion at the early stage will be detected only with highly sensitive CRP assay. In case of preterm neonates, who respond poorly due to immature immunological defence mechanism with a slight rise in CRP than term neonates, which cannot be detected by routine CRP thereby resulting in under-diagnosis of neonatal sepsis among preterm neonates can be corrected by using hs-CRP [13,14]. Other reasons could be CRP are elevated in various other non-infective conditions also like perinatal asphyxia, prolonged labour, tissue injury and meconium aspiration in neonates, which has to be ruled out by taking serial measurements of CRP, by specific tests like endotoxin or combining it with other inflammatory markers like PCT, IL-6 assays[15,16].

During the initial stages of sepsis, the bacterial load is too minimal to be detected by culture. However, endotoxin were released by invading bacteria after log phase of its growth, the initiating factor for Gram negative bacterial sepsis; which explains detection of endotoxin in 8 cases out of the 25 culture negative cases studied. The sensitivity and specificity of endotoxin in our study was found to be 84% and 68% respectively when gram positive and gram negative bacteria were taken as a single group and there was a statistically significant increase in endotoxin levels in culture positive cases compared to culture negative cases (p value = 0.000197). However, considering the Gram negative bacterial sepsis alone, the sensitivity and specificity of endotoxin was found to be 100% and 100% respectively. In a study conducted by Wang et al [17], there was a statistically significant increase in endotoxin levels in Gram negative bacterial sepsis compared to culture negative sepsis with a p value of 0.0025 which was consistent with our results.. Ishihata et al [18] showed that there was statistically significant difference in the endotoxin levels when compared between Gram negative sepsis and control group (p value < 0.0001) which was similar to our results.

The limitations of our study include the small sample size, conventional blood culture which has a lower sensitivity and follow up of the cases was also not done to know the prognostic values based on estimated level of Endotoxin and hs-CRP .The study can be improvised by analysing large sample size, using automated blood culture system and proper follow up of cases to further assess the significant role of hs-CRP and endotoxin not only a diagnostic tool , but also as a prognostic marker in neonatal sepsis.

CONCLUSION:

There was a statistically significant contribution of hs-CRP and endotoxin assays in early detection of neonatal sepsis when compared with blood culture of both positive and negative results. The sensitivity and specificity of hs-CRP were 100% and 76%, whereas the sensitivity and specificity of endotoxin were 84% and 68%. However, the sensitivity and specificity of endotoxin for Gram negative bacterial sepsis was found to be considerably higher. Though both hs-CRP and endotoxin have their own limitations, their combined use along with the other inflammatory markers may pave the way in the future for a definitive and early diagnosis of neonatal sepsis, which still remains to be every pediatrician's nightmare.

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