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ROLE OF INTERLEUKIN-6 AND INTERLEUKIN-10 IN EARLY DIAGNOSIS OF NEONATAL SEPSIS,A PROSPECTIVE STUDY IN A TERTIARY CARE HOSPITAL

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ABSTRACT Neonatal sepsis encompasses overwhelming infection without much localization (septicaemia) or infection localized to lungs (pneumonia) or meninges (meningitis).Early manifestations of neonatal sepsis are often subtle & non-specific. There is no rapid and reliable test for confirmation of etiological diagnosis. Treatment is generally started when clinical picture is supported by indirect early markers of neonatal infection. Many recent studies considered Procalcitonin to be a superior marker of neonatal sepsis compared to C-reactive protein (CRP) with the added advantage of differentiating bacterial & viral infection. Though IL-6 is a very early marker & major inducer of CRP , its level become normal even if infection continues, giving a false-negative result. But the simultaneous determination of IL-6 & CRP can obviate this problem, and sensitivity becomes 100%.

Summary : The objective of this study is to establish clinico-pathological co-relation between neonatal sepsis & cytokines. Specific objectives of this study are to evaluate IL-6 & IL-10 as an early marker of neonatal sepsis and to compare the reliability of IL-6 & IL-10 with other marker of neonatal sepsis, viz.CRP (C reactive protein) in both probable and proven case of sepsis. Both inborn and outborn babies with & without signs and symptoms of neonatal sepsis were included in the study. IL-6 & IL-10 measurement done using immunoassay techniques in the Department of Biochemistry of Institute of Child Health, Kolkata. Blood cultures were positive in 10 cases out of total 63 neonates (16%, most common organism was Klebsiella sp). IL-10 & CRP concentrations are found significantly higher in the sepsis group compared to no-sepsis group. The sensitivity, specificity, positive predictive value, negative predictive value of serum IL-6,serum IL-10 & serum CRP was done using a cut-off value of 19pg/ml, 25pg/ml & 2mg/ml respectively. Combined IL-10 + CRP at lower cut off value has a better sensitivity (94.6%) compared to IL-6 + CRP (91.5%), whereas IL-6 + CRP has a better specificity (33.9%) than IL-10+ CRP (31.2%) . At higher cut off value IL-10+ CRP has better sensitivity and specificity than IL-6 + CRP. Routinely used sepsis markers like TLC, I/T ratio, ANC etc. were found to be inadequate to screen for sepsis on presentation in NICU. IL-10 & IL-6 may be a valid & early predictive marker of neonatal sepsis.

KEYWORDS : Neonatal sepsis, Interleukin-10, Interleukin-6, C-reactive protein, ELISA

INTRODUCTION:

Neonatal sepsis is a clinical syndrome characterised by systemic signs of infection & accompanied by bacteraemia in the first month of life [1,14]. Empirical antibiotic therapy in suspected cases , however renders many neonates unduly susceptible to side effects of antimicrobial agents, increases hospital stay, cost & promotes the development & spread of resistant bacterial strains [2].Positive sepsis screen include(two of 4 parameters) TLC <5000/cmm, Band cell to total polymorph ratio of >0·2, Absolute neutrophil count <1800/c mm, CRP>1mg/dl; and micro ESR >10mm 1st hour. Pro-inflammatory cytokines increase early in neonatal bacterial infection before a rise in CRP can be observed [3,7].Il-6,IL-1 & TNF-alpha are responsible for the release of acute phase proteins (also CRP) during systemic infection [4].The anti-inflammatory cytokines i,e. IL-4,IL-10 & IL-13 inhibit the release of proinflammatory cytokines (Il-6,IL-1 & TNF-alpha,IL-8) by activated macrophages [5]. Therefore markers are needed that reliably identify infected neonates very early [6] .The increased serum levels of IL-1,IL-6,IL-8 & gamma-interferon were found to correlate with the severity & mortality in the course of sepsis [8,9].The present study is based on the suggested hypothesis that cytokines are hormone-like substances & highly potent intercellular messengers, that regulate immunological & inflammatory host response [15]. IL-10 is an anti-inflammatory cytokine, exerts pleotropic effects on the immune system. The patient response to infection & clinical outcome involves a balance between pro-inflammatory & anti-inflammatory cytokines.

In case of ascending infection, pathogenic bacteria mostly do not usually get access until the membrane ruptures. Amnionitis is polymicrobial in nature, most common organisms being *Bacteroides sp.*, Group B *Streptococcus*, other aerobic *Streptococci*, aerobic gram negative bacteria & genital *Mycoplasma* [16]. Longer than 18 hours of membrane rupture is the appropriate cut off for increased risk of neonatal infection [17]. Aspiration or ingestion of bacteria of amniotic fluid may lead to congenital pneumonia or systemic infection with manifestation becoming apparent before delivery (fetal distress, tachycardia), at delivery (perinatal asphyxia), or after a latent period of few hours (respiratory distress, shock). During delivery, the fetus may come in contact with pathogenic bacteria in birth canal for the first time & get infected (Levies 1971)[18]. Organisms like *Listeria monocytogenes* or *Neisseria gonorrhoeae* residing in chronic cervical lesions of the mother or mother being asymptomatic carrier of Salmonellosis & enteropathogenic coliforms may cause infection in the newborns [19].

Newborn infants are relatively immunodeficient host because of their immature host defence mechanism both qualitatively & quantitatively (Jeffery 1977; Edward M.S.1984;Chandra R.K.1984).IgG is actively transported across the placenta. These passively transferred antibodies in adequate concentration provide protection against tetanus & group B Streptococcus infection. Specific bactericidal and opsonic antibodies against enteric gm-ve bacteria are predominantly of IgM class & due to absence of IgM in neonate (as they do not cross placenta),they are susceptible to infection with gram-ve organisms(E.coli & other enterobacteriaceae).Neonate do not synthesize IgA until 2-5 weeks after birth & do not get IgA by transplacental transfer, but may receive it from breast milk in low concentration. In newborn the Polymorphonuclear neutrophils(PMN) exhibit less chemotactic activity than adults.This may be due to marked decrease in membrane deformability of the newborn PMN & deficiency of chemotactic factor C3,C5 (Millar 1971)[20].

IL-6 is synthesized by monocyte, endothelial cells and fibroblasts after stimulation by TNF & IL-1.In contrast to CRP, IL-6 is a very early marker, but levels can become normal even if infection continues. this leads to an increasing false-ve finding when testing is performed later in the course .The simultaneous determination of CRP can obviate this problem (combined sensitivity becomes 100%).

Il-10 is a pleotropic cytokine produced by activated subpopulations of T cells,B cells,monocyte-macrophages,keratinocytes.Il-10 inhibits the production of IL-1,IL-6.

MATERIALS & METHODS:

The study was conducted during the period between July 2013 to June

2014 at Institute of Child Health, Kolkata.Total 451 newborn admitted,63 cases of suspected sepsis were taken for the study purpose. Babies with congenital heart disease, proved case of congenital infection, proven inborn metabolic disorder; were excluded from the study. Simple random sampling was done.

IL-6 & IL-10 measurement done using immunoassay techniques. The neonates were categorized on the basis of their clinical presentation, complete blood count (CBC), C-reactive protein (CRP), and blood culture into: cases (definite infection or clinical sepsis) & controls (physiologic hyperbilirubinemia, negative sepsis work up and uncertain sepsis) .Further categorisation was done according to evidence of respiratory compromise, GI compromise, neurological symptoms, cardiovascular compromise, signs of sepsis & general clinical features(Table 1).

Demography of study population was prepared based on detailed histories including antenatal, natal, postnatal history were taken from all patients . Serum CRP concentrations were estimated using a high sensitivity immunoturbidimetric method on Roche Integra 400 plus auto analyzer (cut off value 5 mg/dl).

Serum IL-6 was measured by the electrochemiluminescence immunoassay "ECLIA" method on cobas e411 immunoassay analyzer. The assay has a measuring range of 1.5-5000 pg/mL and samples with higher concentrations were appropriately diluted. Quality control runs were carried out at regular intervals with satisfactory performance.

For measurement of IL-10, an IL-10 enzyme-linked immunosorbent assay kit (Quantikine human IL-10,R&D Minneapolis ,MN,USA)was used. Cytokine levels are calculated based on standard curves.

STATISTICALANALYSIS :

All clinical data as well as laboratory data of the patients studied were separately compiled and transcribed onto Excel data sheets from the study forms. Statistical analyses were performed with MedCalc Statistical Software version 13.1.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014) . The Kolmogorov–Smirnov test was used to assess sample distributions. The results are presented as the median and 25th/75th percentiles (inter quartile range, IQR).ACR data was found to be non-normally distributed. To compare two independent samples, the Mann–Whitney U-test was used. The chi-square test or the Fisher's exact test was used to compare proportions.

When the distribution of variables is not normal, the degree of association between the variables can be calculated using Rank correlation. The nonparametric Spearman ranked sign procedure was used to assess the significance of associations. The ranked Spearman statistic and the associated p value are presented. In all of the above tests, if the resulting P-value is small ($P<0.05$) then it can be accepted that the median of the differences between the observations is statistically significant different from 0.Therefore, $p < 0.05$ was considered significant.

The diagnostic performance of a test or the ability of a test to discriminate diseased cases from normal cases is evaluated using Receiver Operating Characteristic (ROC) curve analysis. A particular cut-off point or criterion value on the curve was defined to discriminate between the two populations. The discriminating power of this cut-off is reflected by the sensitivity, specificity, positive predictive value . A cut-off value with a higher sensitivity & NPV indicates that a test is ideal for the purpose of screening.

RESULT & ANALYSIS:

Among the total 63 cases, 48 (76%) cases were 0 -7 days old & 15(24%) cases were >7 days old. In this study males outnumber female in a ratio of 2.1:1. Percentage of preterm and term neonate were 33% (21 cases) & 67% (42 cases). Blood cultures (in Brain heart infusion broth) were positive in 10 cases out of total 63 cases (16%).Most common organism grown in blood culture was *Klebsiella spp.* in 3 cases, followed by *Staphylococcus aureus* in 2 cases; *E.coli*, pseudomonas *spp*, *Burkholderia cepacia* in 1 case each & *candida sp* (nonalbicans) in 2 cases. The most common presenting feature observed in this neonates was feed intolerance (66%) followed by lethargy(60%),whereas most common clinical signs elicited was respiratory distress (30%)followed by hypothermia(11%).

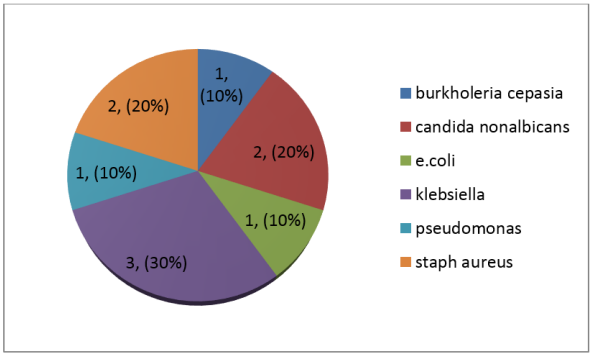

Figure-1: percentage of newborn with organisms isolated in culture

Table -1: Major clinical characteristics on presentation.

Feed intolerance	42 (66%)
Lethargy	38 (60%)
Jaundice	25 (39%)
Respiratory distress	19 (30%)
Irritability	14 (22%)
Hypothermia	7 (11%)
Apnoea	5 (7%)
Abdominal distension	4 (6%)
Hypotension	4 (6%)
Ventilation	4 (6%)
Fever	3 (4%)
Seizure	3 (4%)
Cyanosis	2 (3%)
History of prior antibiotics	0

Table 2: Comparing clinical and laboratory data of patients with and without sepsis with their p-value.

Demography	Sepsis (n= 35)	No sepsis (n= 28)	P value
Mean Age, days (SD)	5.7 ± 1.3	4.4 ± 0.9	0.45
Gender, males (%)	25 (69%)	18 (67%)	0.81
Mean Gestational Age, weeks (SD)	37.3 ± 3.4	37.5 ± 3.9	0.84
Mean Birth Weight, kg (SD)	2.4 ± 0.65	2.5 ± 0.61	0.79
Mean Weight on Presentation, kg (SD)	2.3 ± 0.60	2.4 ± 0.56	0.51

Laboratory parameters	Sepsis (n= 35)	No sepsis (n= 28)	P value
Median White Blood Cell count (10^9/L)	10.35 (8.25 –12.5)	9.6 (7.95 –12.12)	0.69
Median Neutrophils, %	57.5 (42.5 – 65.5)	48.0 (38.5 – 58.7)	0.11
Median Band cells, %	3 (2-5)	3(2-4)	0.38
Median I/T ratio	0.05 (0.03 – 0.08)	0.05 (0.03 – 0.1)	0.86
Median Platelets ($1000/m^3$)	180 (118.5 – 292)	236 (180 – 294.5)	0.13
Median ESR (mm/hour)	8 (5 – 22)	6 (4 – 8)	0.06
Median serum CRP (mg/L)	6.0 (1.8- 35.9)	1.2 (0.4 – 4.8)	0.007
Median serum IL - 6 (pg/ml)	48.1 (23.8 – 162.6)	48.1 (23.8 – 162.6)	0.08
Median serum IL-10 (pg/ml)	33.1 (19.6 – 74.4)	20.9 (14.0 – 31.3)	0.01

Intervention	Sepsis (n= 35)	No sepsis (n= 28)	P value
IV fluids	32 (88%)	10 (37%)	0.06
Oxygen requirement	28 (77%)	7 (26%)	0.03
CPAP requirement	5 (14%)	1 (4%)	0.39
Mechanical Ventilation	4 (11%)	0	0.14
Vasopressor requirement	3 (8%)	0	0.26
Blood products	6 (16%)	0	0.07

Outcome	Sepsis (n= 35)	No sepsis (n= 28)	P value
Mean Total antibiotic received, days (SD)	10.2 (±4.9)	2.2 (±3.3)	<0.0001
Median Total length of NICU stay, days (IQR)	10 (7.5 – 15)	6 (5-7)	<0.0001
Death	0	0	-

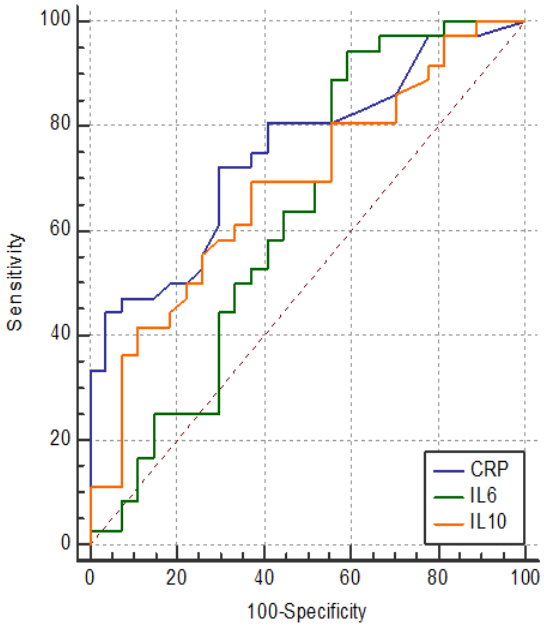

Figure-2: ROC- CURVE :Comparison of Receiver Operating Characteristic (ROC) curves of CRP, IL-6, and IL-10 to predict sepsis in patients admitted to neonatal intensive care unit. CRP had the highest area under the curve (0.75) followed by IL-10 (0.68), and IL-6 (0.63).

Table 3: Sensitivity, specificity, positive- (PPV) and negative-predictive value (NPV) of on-admission values of IL-6, IL-10 & CRP

	Sensitivity %	Specificity %	PPV %	NPV %
IL6 > 25.3 pg/ml	69.4	48.2	64.1	54.2
IL6 > 153.2 pg/ml	25.0	85.2	69.2	46.0
IL10 >18.8 pg/ml	80.56	44.44	65.9	63.2
IL10 >53.2 pg/ml	36.1	92.6	86.7	52.1
CRP >2.2 mg/L	72.2	70.4	76.5	65.5
CRP >9.1 mg/L	44.5	96.3	94.1	56.5

Table-4: comparing sensitivity, specificity, PPV & NPV of IL6, IL10 & CRP with their increasing concentration.

	Sensitivity %	Specificity %	PPV %	NPV %
Il6 > 25 pg/ml	69.4	48.2	64.1	54.2
IL10 > 19 pg/ml	80.6	44.4	65.9	63.2
CRP > 2 mg/L	72.2	70.4	76.5	65.5

	Sensitivity %	Specificity %	PPV %	NPV %
IL6 > 150 pg/ml	25.0	85.2	69.2	46.0
IL10 >50 pg/ml	36.1	92.6	86.7	52.1
CRP >9 mg/L	44.5	96.3	94.1	56.5

With increasing concentration specificity & PPV increases in all the parameters whereas the sensitivity & NPV decreases.

Table-5 : Sensitivity & specificity of IL-6+CRP & IL-10+CRP

	Sensitivity %	Specificity %
IL6(>25.3pg/ml) +CRP(>2.2mg/L)	91.5	33.9
IL10(>18.8pg/ml)+CRP(>2.2mg/L)	94.8	31.2
IL6(>153.2pg/ml) +CRP(>9.1mg/L)	58.4	82.0
IL10(>53.2pg/ml) +CRP(>9.1mg/L)	64.5	89.2

Combined IL-10 + CRP at lower cut off value has a better sensitivity (94.6%) compared to IL-6 + CRP (91.5%), whereas IL-6 + CRP has a better specificity(33.9%) than IL-10+ CRP (31.2%), but at higher cut off value IL-10+ CRP has better sensitivity and specificity than IL-6 + CRP.

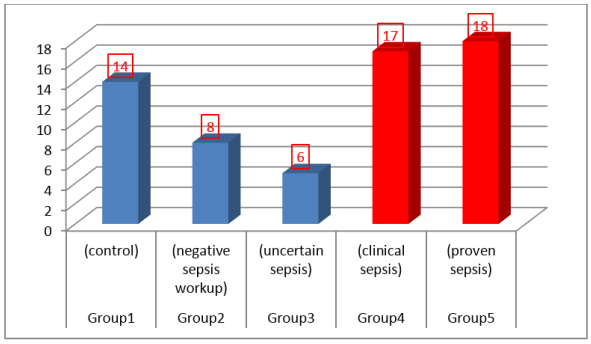

Figure-3: classification of neonates into groups

DISCUSSION:

The present study compared the sensitivity, specificity, positive predictive value & negative predictive value of serum IL-6,Il-10 ,among a study group of 63 suspected neonatal sepsis. Blood cultures were positive in 10 cases, Klebsiella sp being predominant. The IL-10 assay done at the presentation of signs and symptoms in the present study showed that there was a negative predictive value of 63.2% using a cut-off value of 19 pg/ml. More importantly, anti-inflammatory cytokines such as interleukin-10 have counter-regulatory properties that can down-regulate the release and effect of proinflammatory mediators and this may partially explain increased IL-10 in our infected infants.

Several studies have demonstrated that in experimental models of sepsis, supraphysiologic doses of recombinant IL-10 are protective against mortality and proinflammatory cytokine production[10]. IL-10 knockout mice or mice injected repeatedly with antibody to IL-10 showed higher plasma proinflammatory cytokine levels and higher mortality rates following endotoxin injection. Consistent with our finding, plasma levels of IL-10 in animal model correlate with disease severity indices, and in sepsis, with plasma levels of proinflammatory cytokines.In human study high blood concentration of IL-10 were positively correlated with the severity of the infective process and the occurrence of septic shock and multi-organ failure and, in general, signified a poorer prognosis [11] . However, this study was done in an older group of children than in present study.

In the present study we have found that serum IL-10 and CRP concentrations are significantly higher in the sepsis group compared to no-sepsis group. The median value of IL-10 in sepsis group is 33.1(19.6-74.4), and in non sepsis group 20.9(14.0-31.3) with a p-value 0.01. The median value of serum CRP in the sepsis group is 6.0(1.8-35.9) and in the non sepsis group it is 1.2(0.4-4.8) with p-value of 0.08. Whereas median serum IL-6 concentrations are higher in the sepsis group, that is 48.1(23.8-162.6) and in the non sepsis group 28(10.4-130.4), but it is statistically non significant (p=0.08).

IL-10 concentrations and the peak CRP concentrations had sensitivities of 94.8%, Specificity 31.2% in the early diagnosis. It has been reported previously that when using CRP > 1 mg/dl as the cut-off value, the range of reported statistical outcomes is as follows: sensitivity 70% to 93%; specificity 41% to 98%; positive predictive accuracy 6% to 83%; and negative predictive accuracy 97% to 99% [13]. In the present study, serum CRP concentrations correlated positively with serum IL-10 concentration, and the specificity and PPV of the 2 assays were comparable.

CONCLUSION:

In spite of the advances in obstetric care, neonatal care and antimicrobial therapy over last few decades' neonatal sepsis continues to be a major factor causing neonatal morbidity and mortality. Although blood culture is gold standard for diagnosis of neonatal sepsis, it's sensitivity is less due to biasing factors like, previous use of antibiotics, sample size for culture & culture technique; also time taken for result is longer. Newer and faster radiometric methods of blood culture are not available in many centers of India and are also costly. Other routinely used sepsis markers like TLC, I/T ratio, ANC etc. were found to be inadequate to screen for sepsis on presentation in the NICU. IL-6 was not a very robust marker in our setting of a heterogeneous population consisting of both early onset and late onset sepsis, most likely because, Il-6 is a cytokine that increases rapidly with onset of infection and falls to lower levels within a short span. Our

inclusion of late onset sepsis may have influenced our findings. However, Il-10 proved to be a better discriminator in our study population. Its combination with CRP considerably improved the sensitivity and PPV, which would be beneficial to screen neonates for sepsis with confidence. This would help to diagnose sepsis early on presentation whenever the neonatologist has a clinical suspicion of sepsis.

In conclusion, IL-10 may be a valid and early predictive marker of neonatal sepsis and IL-6 can be can be an early predictive marker of early onset neonatal sepsis. Measurement of combined IL-10 and CRP in suspected newborn infection may be useful in triaging patients into those who would benefit from the antibiotic treatment.

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