Annotation

Journey from Cell count to Metabolomics in the diagnosis of Neonatal Sepsis.

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Abstract:

Neonatal sepsis remains a global challenge due to its high mortality and morbidity. One of the reasons for the high loss of neonatal life is the lack of early objective diagnostic biomarkers. The commonly routine tests used currently lack diagnostic certainty. However, emerging technologies like proteomics and metabolomics hold promise of early and specific diagnosis of neonatal sepsis.

This review examines the robustness of the commonly used biomarkers and identifies the developing biomarkers that have the potential to overcome the challenge of early diagnosis of neonatal sepsis.

Keyword: Neonatal sepsis, biomarkers

Introduction:

Neonatal sepsis is a devastating disease and is one of three most common causes of neonatal mortality and morbidity globally. It is estimated that 3 million newborns suffer from sepsis every year 1 , of these between 28 and 36% die (43% dying within the first week of life). The burden of sepsis and the high loss of neonatal life is mainly in the resource poor developing countries. As these countries lack surveillance and registration ability the actual numbers maybe higher.

Since bacteria were recognized to be the source of infection in man around 1860 and that early diagnosis improves outcome efforts have been made to diagnose infection/sepsis as early as possible using a variety of investigative tools.

This review aims to take the reader on a whirlwind journey from white cell count as a marker of infection to the present where metabolomics are being touted as the new diagnostic biomarkers of infection/sepsis.

Prior to embarking on this journey, it is important to clarify certain definitions used in this review;

What is sepsis? Sepsis has been variously defined. For this review the definition has been adopted from the third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) 2 as "A life threatening clinical condition caused by the invasion into a normally sterile tissue, fluid or body cavity by pathogenic or potentially pathogenic organism leading to organ dysfunction caused by a dysregulated host response to infection". Though the consensus definition applies to adults, due to lack of a similar consensus definition for neonatal sepsis most authors in neonatal literature have adopted this definition.

In the newborn sepsis is classified into Early Onset Sepsis (EOS) that is sepsis that occurs within the first 72 hours after birth reflecting vertical transmission usually from the maternal birth canal and Late Onset Sepsis (LOS) that is sepsis that occurs after 72 hours of birth reflecting horizontal i.e. environmental transmission of infection.

2. Why is neonatal sepsis important? Neonatal sepsis is second only to birth asphyxia and prematurity as a cause of neonatal mortality, morbidity and a major problem, especially in developing countries. In developing countries, sepsis is diagnosed in 49 – 170/1000 live births with culture proven-sepsis is 16/1000 live births and neonatal meningitis between 0.8-6.1/1000 live births. Despite many efforts to reduce this high loss of neonatal life from neonatal sepsis in developing countries has proven challenging. One of the main reasons for this is due to lack of highly sensitive and specific biomarker for early diagnosis of neonatal sepsis.

3. Why perform diagnostic tests? With the high degree of uncertainty associated with the early diagnosis of sepsis and the lack of "Gold Standard" amongst the available biomarkers clinicians undertake investigations either for psychological reassurance that either normal or normal results provide, or because that is the policy or for want of "doing something" or that the family feels that the doctor is "doing something" and rarely for legal or financial reasons. However, emerging technologies linked with better understanding of the developing host defense systems to early infection have provided opportunity to develop newer biomarkers (e.g. genomics/proteomics/metabolomics) to improve the diagnosis of neonatal sepsis.

4. How to choose the tests wisely? With the plethora of tests available clinicians need to choose the tests they request wisely. The choice they make is often to use tests that have high sensitivity (always positive in disease in question…… sensitivity is used to rule OUT disease) and high specificity (always negative in healthy

patient …. high specificity is used to rule IN disease). Clinicians often also use 'likelihood ratio' a test that approach 100% to determine their choice. Table 1.

POSITIVE LIKELIHOOD RATIO {*POSITIVE PREDICTED VALUE (PPV)*} Probability of patient **with** disease having a positive test Probability of patient **without** disease having a positive test NEGATIVE LIKELIHOOD RATIO {*NEGATIVE PREDICTED VALUE (NPV)*} Probability of patient **with** disease having a negative test Probability of patient **without** disease having a negative test

Table 1. Definition of Likelihood Ratio (PPV and NPV).

The properties of an ideal diagnostic biomarker include high sensitivity and negative predictive value (approaching 100%) as well as high specificity and positive predictive value. Unfortunately, currently there are no tests that approach 100% sensitivity, specificity or PPV or NPV because in sepsis there is significant overlap between positive and negative tests thus, there remain large area of uncertainty. Figure 1.

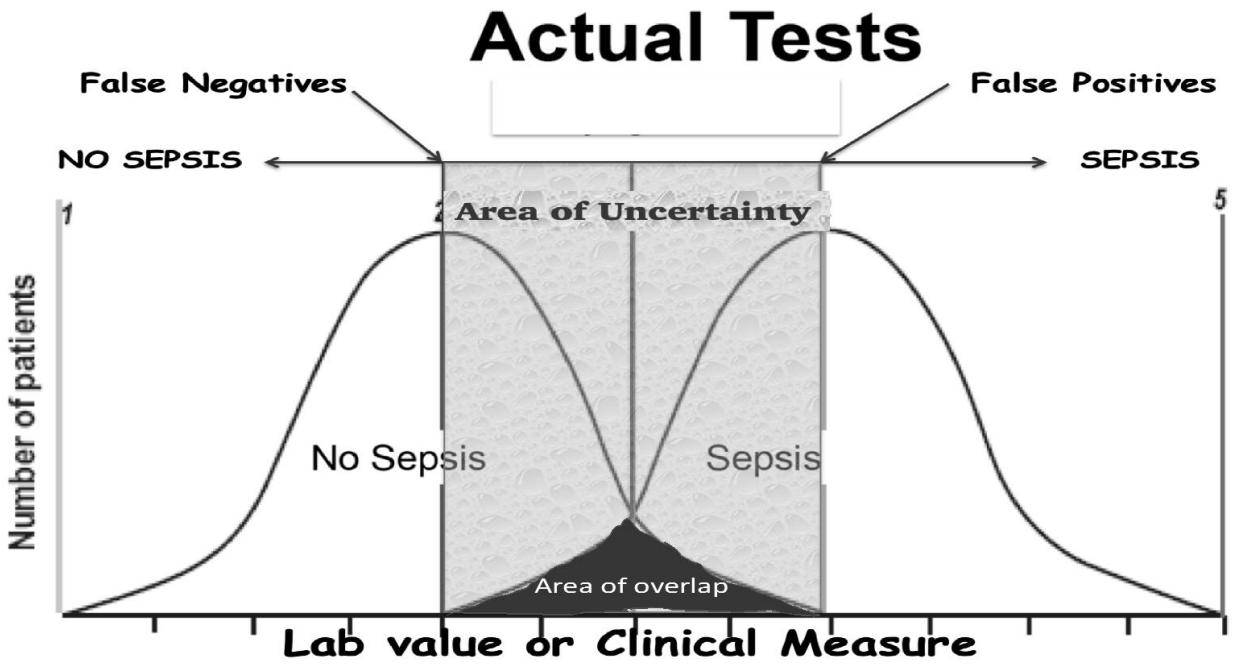


Figure 1. *Varying thresholds results in trade-offs between false positives and false negatives. Showing the areas of overlap and uncertainty.*

Having laid out the background, I would like to discuss individual tests to diagnose neonatal sepsis in order of frequency they are used by clinicians.

Blood Culture: Blood culture is still considered the 'gold standard'. However, it is often unreliable due to intra-partum antibiotics have been administered to the mother.

Blood culture frequently fails to detect bacteremia in 27%-92% of preterm VLBW infants often due to the volume of blood inoculated into the blood culture bottle being insufficient or suboptimal processing of the specimen. In addition, the bacteremia in the newborn is often transitory or intermittent or as a recent study has shown that nearly half of septic infants have low-level bacteremia ($\leq$1 Colony forming units (CFU)/ml). Yield from blood culture does not improve by sending repeated small volume samples but can be increased by inoculating a minimum of 0.5-1 ml of blood into the blood culture bottle. Further difficulty with blood culture is its 'turn around' time of at least 24 -48 hours and in some resource limited countries up to 10 days; this is too long for a test on which clinical decisions to be made. Recent automated culturing systems based on presence of CO2, or pH provide higher degree of accuracy and a 'turn around' time between 12 and 36 hours[3]. To diagnose true CoNS (coagulase negative staphylococcus) infection from contamination on blood culture is challenging. If there is such concern then a central and peripheral blood culture should be drawn. If both cultures are negative then there is no infection, if one of the cultures is positive then contamination is most likely, only if both cultures are positive then it should be taken as true CoNS infection.

White blood Cell Count and Ratio's: Cell counts are difficult to interpret in the neonatal period because they vary significantly with gestation and day of life. Leukocyte number, character and indices are most frequently utilized to diagnose or monitor sepsis. In nearly 50% of infants with infection their values may be normal during the initial phase of infection only to become abnormal after 6 - 12 hours or so[4]. Total leukocyte counts below 4000 x 109 /l, absolute neutrophil count below 1500/mm^3 and immature to total neutrophil (I/T) ratio > 0.24 is associated with early onset sepsis whilst high or low total white cell count, high absolute neutrophil count and high I/T ratio along with low platelet count (< 147,000/mm^3) are associated with late onset sepsis. However, low white blood cell count, absolute neutrophil count, and high immature-to-total neutrophil ratio were associated with increasing odds of infection, but no complete blood cell count-derived index possesses the sensitivity to reliably rule out sepsis in neonates[5,6].

Acute Phase Reactants (Proteins): Acute-phase proteins (APPs) are a class of proteins whose

plasma concentrations increase (positive acute-phase proteins) or decrease (negative acute-phase proteins) in response to inflammation. This response is called the *acute-phase reaction* (also called acute-phase response).

C-Reactive Protein (CRP) is the most frequent acute phase reactant used to either diagnose or monitor sepsis. CRP is synthesized by the liver following IL-6 activation; it is involved in coagulation and opsonization. CRP increases late in infection, with a lag time between 12-24 hours explaining the low sensitivity (60%) in sepsis that increases to 89% by 48 hours

after the onset of sepsis. Specificity and NPV also improve with time reaching 99%-100% by

48 hours of onset of infection[7]. It must be remembered that neonate's capacity to produce CRP is lower than that of an adult. For neonates' different cutoff points have been used, ranging from 0.2 – 95mg/l. The most commonly cutoff used is 10mg/l. A single value of CRP has unacceptably low sensitivity to diagnose sepsis

taking serial levels over 24 – 48 hours improves sensitivity and specificity. We and others have found serial measurements of CRP more helpful in determining duration of antibiotic therapy rather than its ability to diagnose sepsis[3].

Procalcitonin (PCT): PCT is a 14-kDa protein that rises within 4 hours following onset of infection with a half-life between 22 and 29 hours sometimes longer in sepsis making it a very appealing biomarker. It is produced by monocytes and the liver. Diagnostic utility of PCT in early onset sepsis is limited due to endogenous postnatal surge of PCT after birth peaking at 24 hours of postnatal age. Though PCT has an overall sensitivity (81%) and specificity of (79%) it is lower in early onset sepsis but some small studies have reported a sensitivity of 92%, specificity of 97%, PPV of 94% and NPV of 96%[8]. Most studies use a cutoff between 0.3 – 2.5ng/ml for the diagnosis of sepsis. PCT used in conjunction with CRP increases the PPV to 92%.

Serum Amyloid A (SAA): An acute phase apolipoprotein induced by IL-1, TNF-α and IL-6 in the liver, endothelial cells, monocytes and smooth muscle cells. It increases by 8-24 hours after onset of infection and has a sensitivity of 96% with a NPV of 99%. Though more robust than many other acute phase reactants larger studies are required to establish this as a routine test in neonates. Its use is also limited as SAA levels are affected by hepatic function and nutritional status of the infant.

Serum Calprotectin: Serum calprotectin also known as Migration Inhibitory Factor Related Protein (MRP8/14,) or calgranulin-B is a complex of S100A8 and S100A9 protein molecule that is actively secreted via the paracrine and autocrine mechanisms in phagocytic and endothelial cells during stress and inflammation. In late onset neonatal sepsis, it has a sensitivity 89% and a specificity of 96%. The cutoff value is > 1.7μg/ml. Calprotectin is an early, accurate and easy-to-measure/use marker of neonatal sepsis[9].

Other Acute Phase Proteins: There are a large number that have been studied e.g. neopterin,

Lactoferrin, alpha-1-anti-trypsin, anti-thrombin, Inter-alpha Inhibitor Protein (Ialp), Pentraxin 3 (PTX3), LBP, sTREM-1 and others have either not been tested in the newborn or have not gained popularity due to their poor sensitivity, specificity, cost and technical problems.

Cell surface antigens: (CD64, CD 11b, CD14, CD 32, CD 16, CD 69 and soluble CD163). Circulating inflammatory cells such as neutrophils, monocytes, and lymphocytes express cell surface antigens after activation by bacteria. Availability of flow cytometry has made the analysis of cell surface markers possible. CD64 and sCD163 are the most promising markers from this group and have sensitivity between 81%-96% and a NPV between 89%-97%. Whilst promising, estimation of cell surface markers is limited by the need to process blood samples rapidly before neutrophils die from apoptosis or the surface antigens are down regulated.

The soluble fraction of soluble CD 14, sCD14-ST or **presepsin** is highly developed in sepsis and has demonstrated a sensitivity of 94% and specificity of 100% with a negative cutoff value >885ng/l. **Presepsin** has negative likelihood ratio of 0.05 and positive likelihood ratio of infinity[9]. With such excellent sensitivity, specificity, PPV and NPV presepsin perhaps is the best single biomarker of sepsis.

Other markers like CD10, CD11b, CD11c, CD14 and their soluble variants and Monocyte HLA-DR also corelate with sepsis but their sensitivity, specificity, PPA and NPV are unacceptably low.

Cytokines and Chemokines: Cytokines and Chemokines are cellular signaling proteins that play a vital role in response to infection and stress. Recently there has been a flurry of interest in the possibility of using cytokines and chemokines to diagnose and monitor sepsis both in adults and in neonates. Initial measurements of proinflammatory cytokines like TNF-α, IL-2 and Interferon gamma (INFγ) were disappointing due to their very short half-life (~17minutes) leading to high false negative results. Measurements of pro-inflammatory cytokines with longer half-life have (24 hours) proven to be more fruitful. Levels above 70 pg/ml of IL-6 or IL-8 have a sensitivity of 77%-97%, specificity between 76%-97%, a PPV of 42% and NPV of 99% in sepsis. Kauster et al[10] noted that IL-6 actually increased two days prior to clinical diagnosis of sepsis in neonates suggesting that they may be very early markers of sepsis. Chemo-attractant IL-8 with a sensitivity of 92% and specificity of >70%, NPV of 94% appears to be a better marker of neonatal sepsis than IL- 6. IL-8 rises between 2-4 hours of an infection and declines between 4 – 6 hours. Anti-inflammatory cytokines IL-10 and TGF-β which strongly inhibits pro-inflammatory cytokines like TNF-α, IL- 1, IL-6, IL-12 and IL-18 in additions to inhibiting translocation of nuclear Factor-κB.

Anti-inflammatory cytokine IL-10 in combination with IL-6 and RANTES (regulated on activation normal T cell expressed and secreted) have recently been shown to

diagnose disseminated intra-vascular coagulation secondary to sepsis with near

absolute certainty (sensitivity 100%, specificity 97%, and NPV of 100%)[11]. Availability of semi-quantitative cot side measurement of IL-6 requiring only 50μl blood and a 'turn around' time of 20 minutes has brought the prospect of early cot side diagnosis closer.

We (unpublished) using multiplex bead technology have studied an array of cytokines and chemokines in preterm neonates with suspected and proven bacterial sepsis. With a drop of blood (<50 μl) collected on a Guthrie card and a two-hour turnaround time, we have evaluated 20 proinflammatory cyto and chemokines. In this pilot study of 60 infants with culture proven sepsis we found macrophage inflammatory protein (MIP-1ß) to be the most useful diagnostic and prognostic marker with sensitivity of 93% and specificity of 87% and NPV of 98%. This needs to be evaluated further with a larger cohort of infants.

Other cytokines/chemokines like IL-17, IL-3, Monocyte Chemoattractant Protein (MCP-1), High Mobility Group Box protein-1 (HMGB-1), Growth-related Oncogene-alpha (GRO-alpha) and soluble CD40 ligand (sCD40L) have been studied in adults but not in neonates.

The "Omics": The 'omics cycle' includes genomics and met-genomics, transcriptomics and meta-transcriptomics, proteomics and meta-proteomics and metabolomics that aim to identify the pathogens *(who is there),* what are the pathogens doing in the host and establish how they are doing it. Figure 2.

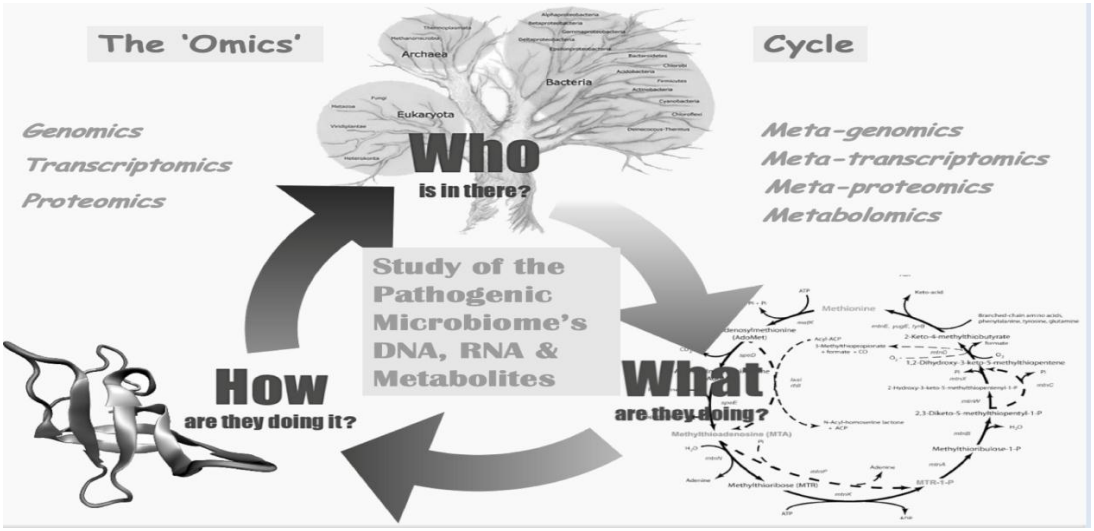


Figure 2. *The 'Omic' cycle.*

Genomics: Gene expression can be studied either by sequencing conserved 16SrDNA and/or meta-genomic sequencing. Genomics detects hosts susceptibility to infection and how the host respond to infection but cannot predict who actually has infection. However, large genomic studies have shown that sepsis related changes are highly variable at transcriptional level hence cannot be relied upon for either diagnosis or prognosis.

Molecular Markers: Emerging molecular techniques using multiplex platforms that can measure multiple proteins, RNA and DNA using techniques like FISH (fluorescence in situ hybridization) and polymerase chain reaction. FISH technique has not been found helpful in neonatal sepsis.

Transcriptomics; Polymerase Chain Reaction (PCR). Transcriptomics is a way of evaluating gene expression in disease. Foremost among these are the 16S rRNA gene which is preserved in all bacteria and 18S rRNA gene is preserved in all candida species. PCR for bacterial 16SrRNA gene gives a sensitivity of 96%, specificity of 99.4%, PPV of 88.9% and NPV of 99.8% . Using microarray hybridization technique PCR not only detects the presence of bacteremia and also identify the offending organism. Thus, PCR has significant advantages over blood culture in that it has much higher accuracy, a short (4-6 hours) 'turn around' time and requires only 0.2-0.3 mls of blood but it is expensive and is not either universally available 24 hours a day. Commercial companies are developing portable machines to do PCR within neonatal units.

Recent study by Mohan et al[12] has shown that in very low birth weight infant (VLBW) with early onset sepsis PCR would miss 2 cases of sepsis and lead to over treatment of 39 without sepsis whilst in late onset sepsis in VLBW infant's PCR would miss 20 cases of sepsis and lead to over treatment of 32 neonates.

Proteomics and Metabolomics: Proteomic and metabolomic technologies try to look at functional expression of proteins in neonatal sepsis whilst metabolomics tries to identify metabolites that are released during infection. The goal of proteomics is to discover every protein expressed in a quantitative and functional manner in any given condition e.g. sepsis and use those proteins or identify protein signature for

diagnosis. Proteomic technology involves separation of proteins based on their intrinsic properties such as molecular weight or affinity to antibodies.

Metabolomic identifies pathogen through Matrix-Assisted Laser Time of Flight Mass spectrometry which provides a window to look at gene and environment e.g. neonatal sepsis. During host and pathogen interaction metabolites are expressed within seconds. These metabolites are functional and dynamic and are unique to any given condition e.g. sepsis. Thus, a library of metabolites provides valuable information about the hosts response to any disease or stress in an ongoing matter. Currently, there are many limitations for this technology to move out of research labs into clinical practice. Nevertheless, the 'omic' technology has a unique potential not only to diagnose disease (sepsis) early but also monitor host-infection interaction and help in determining outcome[13].

Whilst this review has dealt with laboratory diagnosis of neonatal sepsis it would be amiss not to mention heart rate monitoring which has been to shown that decreased heart rate variability is an early indicator of neonatal sepsis. Figure 3.

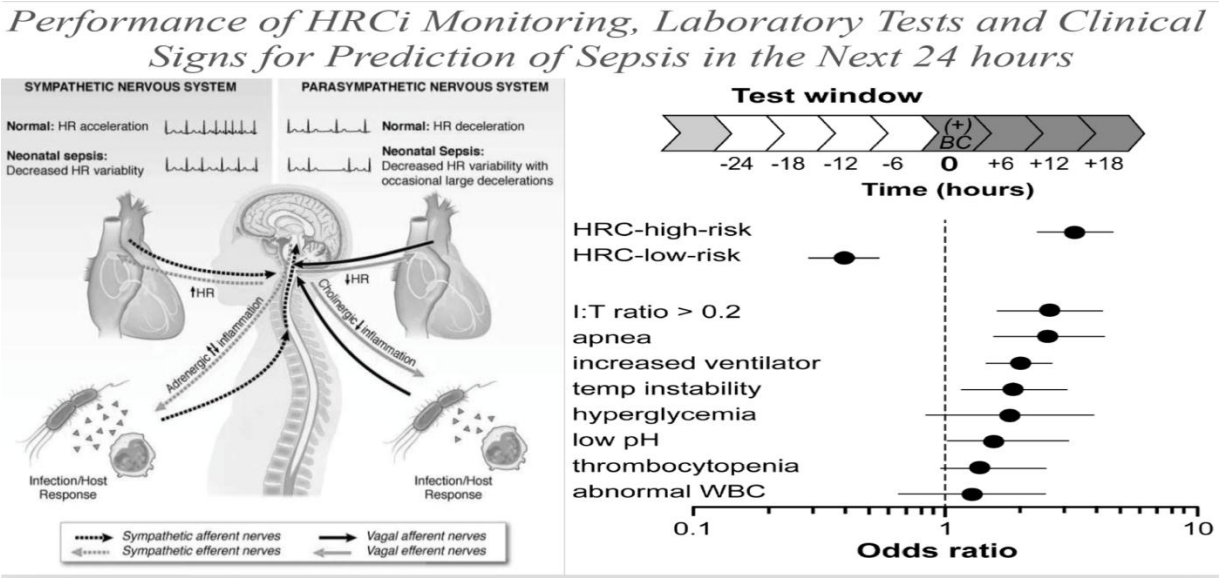
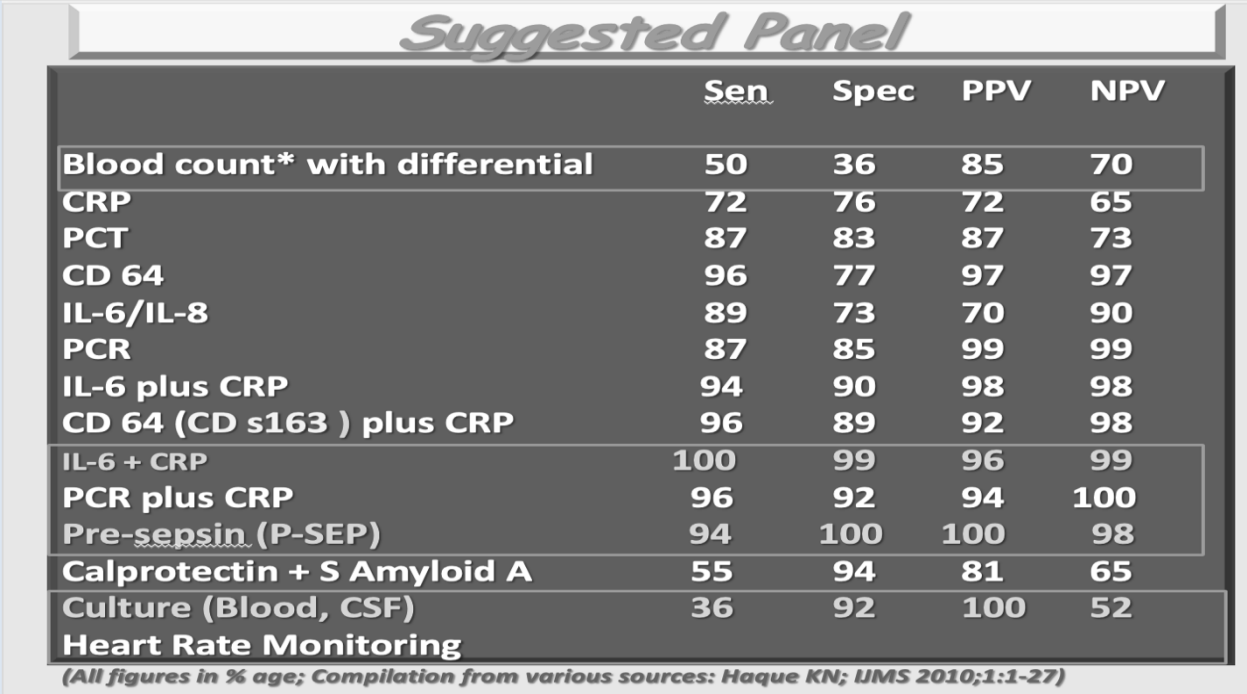


Figure 3. *Decreased heart rate variability is significantly better in the early diagnosis of neonatal sepsis than some clinical and laboratory parameters as shown in the Forrest plot above.*

Conclusion:

The commonly used biomarkers for early diagnosis of sepsis (Haematological parameters, Blood culture, CRP/PCT are unreliable and do not provide the certainty clinicians need. The search for an ideal biomarker or a group of biomarkers that provide early, specific, cost effective diagnosis that is available 24 hours has not yet been identified despite over 200 candidates that have been tested so far. Thus, many authorities have suggested using a panel of tests but there is no consensus on any particular panel or group of tests. One such panel used by the author is given below.



References.

1. Fleischmann-Struzek C, Goldfarb DM, Schlattmann P, Schlapbach LJ, Reinhart K, Kissoon N. The global burden of paediatric and neonatal sepsis: a systematic review. The Lancet Respiratory medicine 2018; 6(3): 223-30.

2. Singer M et al. The Third International Consensus Definitions for sepsis and Septic Shock (Sepsis-3). JAMA 2016; 315(8):801-810.

3. Haque KN. Neonatal Sepsis in the very Low Birth Weight and Preterm Infant: Part 2: Definition, Diagnosis and Management. J Med Scie 2010; 3(1): 11-27.

4. Newman TB et al. Interpreting complete blood counts soon after birth in newborns at risk of sepsis. Pediatrics 2010; 126(%): 903-905.

5. Hornik CP, Benjamin DK, Becker KC, et al. Use of the complete blood cell count in early-onset neonatal sepsis. Pediatr Infect Dis J 2012;31:799–802.

6. Hornik CP, Benjamin DK, Becker KC, et al. Use of the complete blood cell count in late-onset neonatal sepsis. Pediatr Infect Dis J 2012; 31:803–7.

7. Blommendahl J, Janas M, Laine S, Miettinen A, Ashorn P. Comparison of prolactin with CRP and differential white blood cell count for diagnosis of culture proven neonatal sepsis. Scand J Infect Dis 2002; 34: 620-2.

8. Chiesa C et al. Reliability of procalcitonin concentrations for the diagnosis of sepsis in critically ill neonates. Clin Infect Dis 1998; 26(3): 664=672.

9. Pierrakos et al: Sepsis Biomarkers a Review. Crit care Med 2010;14(1): R15.

10. Küster H, Weiss M, Willeitner AE, et al. Interleukin-1 receptor antagonist and interleukin-6 for early diagnosis of neonatal sepsis two days before clinical manifestation. Lancet 1998; 352: 1271-7.

11. Ng PC, Li K, Leung TF, et al. Early prediction of sepsis induced disseminated intravascular coagulation with interleukin-10, interleukin-6 and RANTES in preterm infants Clin Chem 2006; 52: 1181-9.

12. Pammi M, Flores A, Leeflang M, et al. Molecular assays in the diagnosis of neonatal sepsis: a systematic review and meta-analysis. Pediatrics 2011;128: e973–85.

13. Mally P et al. Biomarkers for neonatal sepsis: recent developments. Res and Reports in Neonatology 2014; 4: 157-168.