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“A Pilot study to Evaluate the Utility of placental/Umbilical Cord Blood (PUCB) in Early Onset Sepsis in Very Low Birth Weight Infants”

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1. Introduction and Purpose:

Sepsis is the leading cause of neonatal mortality and every year three million infants die worldwide due to neonatal sepsis (1). In the US, despite significant advances in care, sepsis remains a significant cause of neonatal morbidity and mortality, particularly among Very Low Birthweight (VLBW) preterm infants (PI) (2).

Neonatal early onset sepsis (EOS) is defined as onset of signs and symptoms of sepsis with positive culture <72 hours of age. Compared to term infants, the incidence of EOS is 11 times higher in preterm infants with a mortality rate ranging from 1.5% in term infants to almost 40% in VLBW preterm infants (2).

The diagnosis of neonatal sepsis is challenging, and the prognosis depends on early detection and treatment. The current “gold standard” to diagnose sepsis is a positive blood culture, but there is a variable sensitivity mostly due to inadequate sample volume, transient bacteremia, intrapartum antibiotics and administration of antibiotics before blood sample collection (3,4,5). Although the gold standard for diagnosis of bacterial sepsis is blood culture, only in 25%-40% of blood culture pathogens can be detected (6). Serum markers as IL-6 and CRP have shown to be suitable markers for detecting early onset neonatal sepsis, with a high degree of sensitivity and specificity (18).

In VLBW infants, almost 3 ml of blood is drawn to evaluate EOS for blood culture, CBC and blood group and cross match. This baseline work-up laboratory may represent almost 5-10% total blood volume of a VLBW infant. This iatrogenic acute blood loss may lead to hemodynamic instability and early red blood transfusion in the preterm infant when they are at the highest period risk for intra ventricular hemorrhage (IVH) (23).

The average infant’s blood volume is 80ml/kg, while the average blood in the placenta and umbilical cord is15 to 34 ml/kg which is discarded in the current practice (29). EOS evaluation which includes blood culture, CBC, IT ratio along with biomarkers of EOS like CRP and IL-6 could be done by using placenta/umbilical cord blood (PUCB). PUCB collection is painless, easy and adequate volume may be collected for blood culture. This procedure could decrease iatrogenic blood losses of VLBW infants (23). Currently, there is inadequate published data to support the routine use of PUCB in the evaluation of EOS in VLBW preterm infants.

The purpose of the present study is to evaluate the utility of placenta/umbilical cord blood (PUCB) to perform the baseline workup testing (CBC, I/T ratio, blood culture) along with CRP and IL-6 levels in the evaluation of EOS in VLBW infants.

2. Background:

Early and timely diagnosis of EOS is very important but challenging at the same time as clinical signs are nonspecific. Routine Laboratory tests such as complete white blood count (WBC) with differential count and immature/total neutrophil (I/T) ratio are routinely used to assist in the diagnosis of sepsis in neonates; however, multiple studies have determined that the WBC and immature-to-total neutrophil (I/T) ratio have low sensitivities and specificities (7). The I/T ratio has been found elevated in a quarter to half of the presumptively in infants without sepsis (8,9) with low positive predictive values (7). Consequently, just CBC indices in the evaluation of EOS in neonates is not very helpful, hence use of empiric antibiotic therapy is used in a large number of neonates with clinical suspicion of EOS (8,9).

The “Gold Standard,” blood cultures are not always positive because of low blood volumes are often put in the blood culture bottles and prenatal administration of antibiotics (6, 27). Using biomarkers of sepsis to distinguish septic from non-septic neonates may allow judicious discontinuation of antibiotics, consequently preventing prolonged use of antibiotics and avoiding the appearance of antibiotic-resistant bacteria. Besides, appearance of antibiotic-resistant bacteria, prolonged empiric antibiotic use has been associated with increased risk of necrotizing enterocolitis (NEC), late onset sepsis (LOS), and even increased mortality (10).

Recent studies have shown improved sensitivity and negative predictive value (NPV) of various biomarkers of sepsis compared to CBC indices (11,12). Acute phase proteins, components of the complement system, chemokines, cytokines, adhesion molecules, and cell surface markers have all been investigated as biomarkers of neonatal sepsis (12). The most widely studied and most promising markers include C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-8 (IL-8), procalcitonin (PCT), and tumor-necrosis-factor-alpha (TNF-alpha).

2.1 *C-Reactive Protein (CRP)*

CRP is one of the most sensitive acute-phase reactants for inflammation. CRP is synthesized by the liver. The CRP response frequently precedes clinical symptoms of sepsis. CRP acts within the humoral immune system, particularly in the detection and clearance of bacterial microorganisms. CRP level increases within 6-18 hours after a stimulus and peaks 8-60 hours later (13). The fetus and newborn synthesize CRP; its level is not affected by gestational age, and CRP does not cross the trans-placental passage of maternal CRP (14).

CRP elevations are nonspecific and may be useful for the detection of systemic inflammatory processes; to assess treatment of bacterial infections with antibiotics. In neonates, CRP has been shown to be more sensitive and specific in diagnosing sepsis than the more commonly used CBC indices (total neutrophil count and I/T ratio) (15). Joram at al found that CRP (cut-off point of 5 mg/dl) measured in umbilical cord blood has a sensitivity, specificity, positive and negative predictive values are respectively 50%, 97%, 67% and 94%. Besides, the same study found that the positive and negative likelihood ratios were 16.7 and 0.5 for CRP (28).

2.2 *IL-6*

IL-6 is a pro-inflammatory cytokine produced by mononuclear phagocytes, endothelial cells, and fibroblasts in response to inflammation. IL-6 levels peaks 2-3 hours after inflammatory

responses, and returns to baseline after 6 to 8 hours (16). IL-6 is the primary inducer of hepatic acute-phase protein synthesis, including CRP and fibrinogen (17). IL-6 levels are not influenced by gestational age and has been shown to have high sensitivity in diagnosing EOS (16,18).

Messer et al. in a prospective study, measured IL-6 levels (n=157) upon admission to the NICU and the in umbilical cord blood (n=131) (18). The sensitivity and NPV of IL-6 in the diagnosis of blood culture positive and clinical sepsis was 100% in those infants who had an early measurement of IL-6 at birth (umbilical cord blood) or blood drawn from the infant within one hour after birth. Moreover, they found high sensitivity and NPV persisted until the twelfth hour of age. Krueger et al. found umbilical cord blood IL-6 levels have high sensitivity (87%) and specificity (90%) in 71 term and 100 preterm infants. When only preterm infants were included in the analysis, IL-6 sensitivity and specificity increased to 96% and 94% (19).

2.3 *Combination of Biomarkers*

Because single biomarker cannot be reliably used to diagnose EOS , many investigators have used combinations of biomarkers to improve the sensitivity and specificity in diagnosing EOS. Buck et al. (20) found the sensitivity of IL-6 in CRP-negative newborns and the sensitivity of CRP in IL-6 negative newborns on admission to be 100% in newborns with blood-culture positive or clinical sepsis. They concluded that the combination of IL-6 and CRP is the best combination of biomarkers of sepsis in diagnosis of EOS. Doellner et al. (21) also found the combination of IL-6 and CRP is superior in diagnosing neonatal sepsis than either parameter alone. Laborada et al. also concluded that the combination of IL-6 and CRP is the best diagnostic test for the detection of EOS sepsis in preterm infants (22).

2.4 *Reducing blood withdrawals in preterm infants*

Placental/umbilical cord blood (PUCB) could be used in the evaluation of EOS in PI. (23,26). Christensen found reduced iatrogenic blood loss using PUCB sample during the first day of life ($p < 0.007$), and for the first week of life ($p < 0.001$) in contrast with infant blood sample controls (23). This postpone or eliminating blood transfusion in the VLBW preterm infant (23,26).

PUCB for blood cultures could be a convenient alternative to ensure an adequate volume of blood (24). Meena at al. compared PUCB with blood from infants, (n=40) in the evaluation of sepsis. They found PUCB showed 100% sensitivity and 95% of specificity compared with 100% of sensitivity and 74% of specificity using blood from infants. Beeram et al found that the utilization of umbilical blood samples for CBC and blood cultures are a reliable alternative for sepsis workup in neonates (25).

In the current study, we want to find out the efficacy of PUCB in the evaluation of EOS in preterm infants compared to blood collected from the infant (24).

3. Concise Summary of Project:

Hypothesis: Placental/umbilical cord blood can be used to do early onset sepsis evaluation in low birth weight infants.

Objective: To compare placental/umbilical cord blood and blood from the infant in the evaluation of EOS in VLBW infants.

Specific Aim 1: To compare blood culture, CBC and I/T ratio results of blood from the infant and placental/umbilical cord blood (PUCB).
Objective: All VLBW infants receive empirical broad-spectrum antibiotics after collecting blood for CBC, I/T ratio, and blood culture. We will compare the results of PUCB and blood from the infants, especially comparing white cell count, band count, I/T ratio and if the blood culture is positive or negative. If the blood culture is positive, then we will find out if the micro-organism is the same or not.

Specific Aim 2: To evaluate the role of adding IL-6 and CRP in the evaluation of EOS in VLBW infants.
Objective: We will collect 1 mL of PUCB which will be centrifuged at -40C and serum will be stored at -80 degrees. We will use ELISA to measure IL-6 and CRP. A combination of IL-6 and CRP has been shown to be very helpful in the diagnosis of EOS in VLBW infants. We will also calculate sensitivity, specificity, negative predictive and positive predictive values of IL-6 and CRP individually, as well as, a combination of IL-6 and CRP in the diagnosis of EOS in VLBW infants. We will also find out the duration of antibiotics used in all subjects and find out from IL-6 and CRP data if the antibiotic use was warranted. In future, this may be very useful information in deciding antibiotic duration which will help in avoiding side effects of the unnecessary use of broad-spectrum antibiotics. At present, in our NICU, IL-6 and CRP are not part of EOS evaluation in VLBW infants.

Clinical team will be blinded to the PUCB sepsis evaluation results. In the present study, IL-6 and CRP will not be used clinically but only for research purpose to find out if the addition of IL-6 and CRP help in the management of EOS in VLBW infants.
All VLBW infants get CBC done at admission before starting antibiotics as part of EOS work up. After CBC is done, left over blood sample is saved in the hematology laboratory for 24 hours. We will collect the left-over sample and collect serum after centrifugation. The serum will be saved at -800C for future measurement of IL-6 and CRP. We will compare IL-6 and CRP from infant and PUCB.

Routine management of VLBW infants in NICU at UTMB Galveston:
In NICU at UTMB Galveston, routinely, all VLBW infants admitted to NICU get sepsis evaluation by collecting blood from infant for CBC, I/T ratio, and blood culture followed by a course of empiric antibiotics for 2 – 3 days. This requires almost 2 – 3 mL of blood volume which may be up to 5% blood loss in an extremely premature infant which may lead to hemodynamic instability and anemia, resulting in the requirement for blood transfusion. If the sepsis evaluation is not conclusive, then the course of antibiotics may be extended to 3 to 7 days. Addition of CRP and IL-6 in EOS evaluation may be helpful in determining the duration of antibiotics. However, in NICU at UTMB Galveston, CRP and IL-6 are not part of EOS evaluation.

Placenta/Umbilical Cord blood (PUCB):
In the current research project, we want to use PUCB to do sepsis evaluation in VLBW infants. There is approximately 15 – 20 mL/kg blood in the placenta and umbilical cord. That means in VLBW infants (<34 w) there will be approximately 15 – 20 mL blood in the placenta and umbilical cord which is fetal blood hence it can be utilized to do EOS sepsis evaluation. This

will save blood in VLBW infants. As blood in PUCB is nothing but fetal blood. There is a misconception that fetal and maternal blood is mixed in the placenta. Actually, there is no mixing of maternal and fetal blood in the placenta. After birth, the placenta and umbilical cord are discarded along with the blood inside. We want to utilize fetal blood in the placenta/umbilical cord for the evaluation of EOS in VLBW infants.

The current research project will evaluate the utility of PUCB to assess early onset sepsis (EOS) in VLBW infants (<34 weeks). This study will not affect the management of the infants at all. The lab tests will be done in blocks and hence the results will not be available in real time. The clinical management of infants will be decided by the neonatal team taking care of the infants. The neonatologists will continue to treat infants as per the NICU protocol.

We will compare blood culture, CBC, and I/T ratio results from the infant and PUCB. We will calculate the sensitivity, specificity, positive and negative predictive value of these tests in the evaluation of EOS.

During hospital stay, all VLBW infants get CBC done immediately after birth. After CBC is done, the left-over sample is stored in the hematology laboratory for 24 hours. After 24 hours, this blood sample is discarded. We will collect left over blood from the hematology laboratory to extract serum by centrifuging the blood sample. The serum will be stored at -80^{0}C for future measurements of CRP and IL-6. We will also compare CRP and IL-6 levels in placenta/umbilical cord blood and discarded infant blood. Additionally, we will evaluate the role of adding IL-6 and CRP in the evaluation of EOS in VLBW infants.

Power analysis:
In order to do a power analysis to determine sample size, an estimate of effect size was based upon the Rashawn et al (30) study which found significant correlations of CRP with other biomarkers.
In G*Power (31) the following was used:

- Effect size of r=.4
- Alpha=.05
- Power (1-alpha)=.95
- Yielding a sample size of 63

To accomplish this project, a cohort of preterm infants will be recruited from the infants admitted to the NICU at UTMB, Galveston. We routinely admit around 80 infants/year who are <34 weeks' gestation. We expect to recruit about 50 – 60% VLBW infants born in UTMB, Galveston in the current study. Therefore, we hope to recruit our desired sample size (n=63) in about 18 months.

Clinical Data Collection:
We will use electronic medical records to collect medical data from mother's antenatal, delivery and post-delivery information and detailed medical information of the infants. Data derivated from blood test analysis in placenta/umbilical blood cord and blood from the infants will be used to assess the accuracy of placenta/umbilical blood cord samples. We will also find out specificity, sensitivity, positive and negative predictive values of CBC, I/T ratio, blood culture, CRP and IL-6 in the evaluation of EOS in ELBW infants. All data will be de-identified but the clinical data will be linked to the discarded samples.

4. Study Procedures:

Placenta/Umbilical cord blood (PUCB) sample collection:

After infant is delivered, placenta along with clamped umbilical cord Blood will be obtained from the ObGyn team. One umbilical clamp will be placed at the umbilical end, and the other clamp will be placed on the placental end of the umbilical cord. Then the umbilical cord will be cut between the clamps. The umbilical cord will be cleaned three times with 2% chlorhexidine, plus 70% isopropyl alcohol under sterile conditions (sterile gloves). Cord blood samples will be collected using vacutainer blood collecting system with a sterile 22-gauge needle. We will collect 3 - 4 ml of PUCB.

Discarded blood sample from infants:

During hospital stay, all VLBW infants get CBC done immediately after birth. After CBC is done, the left-over sample is stored in the hematology laboratory for 24 hours. After 24 hours, this blood sample is discarded. We will collect left over blood from the hematology laboratory to extract serum by centrifuging the blood sample at 4^0C for 10 minutes. The serum will be stored at -80^0C for future measurements of CRP and IL-6.

PUCB culture:

After UCB collection, the needle will be replaced by another sterile needle and 1 ml of UCB will be transferred to the blood culture bottle. The culture bottle will be labeled with a unique identification number and immediately will be sent to the microbiology laboratory at UTMB Galveston for blood culture. On identification of positive blood culture, the blood culture bottle will be removed from the machine and will be Gram stained to make sure there are organisms seen and then sub-cultured on blood agar and Mac-Conkey agar plates. The organisms isolated on the culture plates will be identified by VITEK MS (BioMerieux).

Complete blood count and I/T ratio:

After PUCB culture, approximately 1 ml of whole blood will be transferred in an EDTA vial which will be labeled with a unique identification number. The blood sample will be sent to the hematology laboratory at UTMB Galveston for CBC and differential count. I/T ratio will be calculated from the differential count.

5. Sub-Study Procedures:

IL-6 and CRP measurement:

The remainder of the PUCB will be transferred to a heparinized container. PUCB will be centrifuged at 4^0C for 10 minutes to collect supernatants (serum). Serum will be divided into aliquots of 200 microliters each and stored at -80^0C for future IL-6 and CRP measurements. We will measure IL-6 by enzyme-linked immunosorbent assay (ELISA) for human IL-6.

6. Criteria for Inclusion of Subjects:

Inclusion criteria: Subjects who will fulfil following criteria will be included in the study -

1. Infants < 34 weeks' gestational age, born at UTMB, Galveston

7. Criteria for Exclusion of Subjects:

Exclusion criteria: Subjects meeting any of the exclusion criteria at baseline will be excluded from study participation.

1. Known congenital or chromosomal anomalies
2. Congenital heart disease (other than PDA, PFO or ASD)
3. Multiple pregnancy

8. Sources of Research Material:

We are going to use the discarded placenta/Umbilical Cord unit and as the resource of PUCB samples and left-over blood sample after CBC is done as blood from infant.

9. Recruitment Methods and Consenting Process:

Recruitment

All eligible infants born at UTMB Galveston will be recruited in the study. We expect 50 - 60% success in the recruitment after accounting for exclusion criteria and timing of delivery. If the infant is born after hours, the research team may not be available to collect the PUCB sample. We admit around 80 infants/year who are <34 weeks' gestation. We hope to recruit our desired sample size (n=63) in 18 months.

Consenting process

In the current research protocol, we will collect PUCB after birth, which is considered as discarded tissue. We will not collect any blood or any other body fluid from the subjects. This will be less than minimal risk to the subjects. Therefore, consent is not required.

10. Potential Risks:

There is no potential risk to the subjects since PUCB will be collected after birth and placenta and umbilical cord is considered discarded. There will not be any direct interaction with the subjects.

11. Subject Safety and Data Monitoring:

Subject's safety will not be affected as we will be collecting the blood samples from the placenta/umbilical cord after birth which is discarded material. All the data will be collected and saved in a password protected computer. Only PI and research team will have access to the computer and the data.

12. Procedures to Maintain Confidentiality:

No information regarding the study or the clinical data will be released to any unauthorized third party without the prior written approval of the principal investigator. The investigators are committed to maintaining the confidentiality of the subjects through the proper use and disclosure of patients' personal health information (PHI). Study records may be made available for internal compliance reviews and quality assurance representatives.

13. Potential Benefits:

There will be no direct benefit to the subjects. But the results of this study may help us to find out if PUCB could be utilized to evaluate EOS in VLBW infants. The results of this study will provide understanding of the utility of PUBC samples to evaluate EOS in VLBW preterm infants. Moreover, this alternative modality of blood analysis could contribute to the prevention of early hemodynamic instability and early blood transfusion in a very high-risk population of VLBW preterm infants. Furthermore, an additional test for sepsis screening as CRP and IL 6, might improve the accuracy for EOS evaluation, and may avoid or prevent unnecessary and prolong antibiotic use in ELBW infants.

14. Biostatistics:

Statistical analyses will be performed using SPSS 25 for Windows (SPSS Inc., Chicago, IL, USA). Demographic and clinical characteristics will be compared using the non-parametric Mann-Whitney U test, and the results will be presented as medians (range). Categorical variables will be compared using the linear-by-linear association Chi-squared test and will be presented as percentages (%). Receiver operator curve (ROC) analysis will be employed to display the relationship between sensitivity and false positive (FP) rate (1-specificity) and to select the best cut-off value for umbilical cord IL-6 and CRP to predict EOS. Data will be as adjusted by gestational age at delivery, administration of prenatal corticosteroids and antibiotics. Differences will be considered statistically significant at a confidence level of $p < 0.05$ with two-sided alternative hypotheses

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