RESEARCH PAPER

Diagnostic Reliability of Salivary C-Reactive Protein as an Alternative Noninvasive Biomarker of Neonatal Sepsis

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Objective: To assess if salivary C-reactive protein (CRP) can be detected in neonatal sepsis and correlate the levels of salivary and serum CRP. **Methods:** This analytical cross-sectional study included all neonates ≤28 days of life with suspected sepsis or with perinatal risk factors for sepsis. Saliva was collected using an absorbent swab and analyzed by enzyme-linked immunosorbent assay, along with serum CRP. **Results:** Salivary CRP was detectable in 135 subjects (99%). An increase was seen in median (IQR) levels from 0.25 (0.13,0.3) ng/mL in clinical sepsis group to 0.6 (0.3,1.4) ng/mL in screen positive/ blood culture negative group, and to 1.98 (0.54, 2.95) ng/mL in blood culture positive group. There was a moderate positive correlation between salivary and serum CRP (r=0.63, P value 0.01). On receiver-operator characteristics curve, the area under the curve of salivary CRP for predicting serum CRP ≥10 mg/L was 0.861 (95% CI, 0.78 to 0.94; P <0.001), with the optimal salivary CRP cut-off being 0.6 ng/mL. **Conclusion**: Salivary CRP could be used as an alternative biomarker of neonatal sepsis.

Keywords: *Detection, Elisa, Inflammation, Sensitivity.*

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Blood culture is currently the gold standard for diagnosing neonatal sepsis, but various biomarkers in serum are commonly used for rapid supportive diagnosis. Among them, serum C-reactive protein (CRP) is the most extensively studied biomarker in neonates and is found in various body fluids like serum and saliva [1,2]. Considering the difficulty with phlebotomy in neonates and the potential problems caused by blood sampling, alternative fluids like saliva could serve as an attractive non-invasive option for detection of CRP. Salivary diagnostic assays have now been well described in various systemic and oral diseases in adults [3,4]. However, translation into neonatal clinical practice is limited and is mostly restricted to cortisol assessment as a stress marker in term and preterm infants [5,6]. The present study was thus planned to investigate if CRP is detectable in saliva, and whether levels correlate with serum CRP, among neonates with sepsis.

METHODS

This was a cross-sectional study conducted in the neonatal intensive care unit of a tertiary care hospital in over a period of 18 months (September, 2015 to January, 2017). The study was approved by the Institute's research ethics committee.

Neonates (aged ≤28 days) of any gestational age, with clinical suspicion of neonatal sepsis or with perinatal risk factors of sepsis, were included in the study, after informed written consent. Neonates with major congenital malformations, oral infections and oral ulcers were excluded from the study. Demographic findings, including intrapartum maternal fever, presence of preterm and or prolonged rupture of membranes, chorio-amnionitis, gestational age, sex, antibiotic treatment, clinical features, and day of presentation were recorded. All subjects underwent a sepsis workup including serum CRP, complete blood counts, and blood culture, in addition to other investigations as deemed necessary by the treating physician. Serum CRP was measured using immuno-turbidimetric method using Roche Cobas C series analyzer (Roche Diagnostics). Neonates were then further classified into three groups: Group I – blood culture and sepsis-screen positive sepsis, Group II – blood culture negative but sepsis screen positive (serum CRP ≥10 mg/L and one additional parameter - neutropenia based on standardized age based charts or immature: total neutrophil ratios > 0.2), and Group III - only clinical features or perinatal risk factors.

Saliva was collected within 4 hours of collection of the serum sample. Antibiotics were started only after collection of the salivary sample. SalivaBio Infant's Swab

(SIS) (Salimetrics), was used for this purpose [7]. Salivary sampling was done 60 minutes before or after a feed or any oral procedure. The 90 mm SIS was placed between the cheeks and the lower gums after turning the infants head to one side. The swab was left in place for 3 minutes and then removed. The swab was then visually inspected and if found dry, re-introduced for an additional 3 minutes, up to a maximum of two attempts. Only clear saturated swabs were used. The swab was then introduced, saturated end first, into a plunger removed 5 mL syringe and then the re-introduced plunger was used to squeeze out saliva into cryovials. The process was repeated till 0.5 mL volume of saliva was obtained. If sample collected was inadequate, then the saliva collection was repeated with a longer oral stay period for the SIS. In case of any infant discomfort (gag, brow bulge, nasolabial furrow etc.) noticed during procedure, it was immediately discontinued and resumed after signs abated. After collection, labelled cryovials were frozen at –20ºC within 4 hours of collection. The saliva collection was done by the treating team members, after structured training. Each member was supervised during initial few sample collections, to ensure standardization.

On the day of the analysis, saliva samples were thawed and centrifuged. Samples were brought to room temperature before the dilutions. The Salimetrics salivary CRP enzyme immunoassay kit (Salimetrics), an indirect sandwich ELISA kit, was used as per the manufacturer's recommended protocol [8]. All samples were assayed in duplicate, and the average of the duplicates was used in the statistical analyses. Intra and inter-assay coefficients of variation were less than 10% and 15%, respectively. The salivary CRP was estimated in the entire cohort and also in the three groups of infants.

Assuming that salivary CRP has a minimum expected sensitivity and specificity of 75% to predict serum CRP ≥10 mg/L, and an expected prevalence of neonatal sepsis of 40% (as per departmental statistics for the previous year), with an absolute precision of 10% and 90% confidence level, the required sample size was 128 subjects (STATA IC, ver. 13). To account for 5% non-response rate, a total of 136 subjects were included in the final study.

Statistical analysis: Considering the skewed distribution of serum and salivary CRP levels, correlation was assessed using Spearman rank order (Spearman's rho) coefficient. The utility of salivary CRP in predicting elevated seum CRP (≥10 mg/L) was assessed by receiver operating characteristic (ROC) curve analysis and an appropriate salivary CRP cut off value was calculated. The sensitivity, specificity, predictive values and likelihood ratios of salivary CRP at the derived cut-off were calculated for predicting serum CRP ≥10 mg/L and positive blood culture. *P* value <0.05 was considered statistically significant. IBM SPSS version 22 (SPSS Inc.) was used for statistical analyses.

RESULTS

A total of 182 neonates satisfied the inclusion criteria. 32 neonates were excluded because of refusal of consent or already receiving antibiotics. Adequate salivary sample could not be obtained in 9 neonates and in 5, the samples were not analyzed because of contamination. Thus,136 neonates were included in the final analysis. The median (IQR) birthweight was 1.98 (1.34,2.57) kg and gestational age was 34.5 (32,37) weeks. Early onset sepsis (≤3 days) was seen in 88 (64.7%) of the population. Salivary CRP was detectable in 135 (99%) neonates and the levels increased significantly from Group III to Group I neonates (**Table I**).

There was a moderate and statistically significant positive correlation between salivary and serum CRP values in the entire study population (r=0.63; P =0.01) and in Group I (r=0.63; P =0.01) and Group II neonates (r =0.5; P =0.01).

The area under the ROC curve for salivary CRP to predict serum CRP ≥10 mg/L was 0.861 (95% CI, 0.78 - 0.94, P<0.01), indicating good predictive validity. Based on the co-ordinates of the ROC curve, the cut-off of 0.6 ng/mL was chosen as the optimal salivary CRP cut-off value for predicting serum CRP ≥10 mg/L. Salivary CRP ≥0.6 ng/mL had a 77% sensitivity, 94% specificity, 99% positive predictive value and 35% negative predictive value, for predicting a serum CRP level of ≥10 mg/L; and 75% sensitivity, 58% specificity, 44% positive predictive value and 85% negative predictive value, for predicting a positive blood culture.

DISCUSSION

We found that CRP can be detected in saliva of neonates with sepsis, and increases significantly in those with

Table I Serum and Salivary C-Reactive Protein Levels in Neonates (*N*=136)

Variables	*All neonates*	*Group I n=43*	*Group II n=77*	*Group III n=16*
Serum CRP (mg/L)	36.4 (19, 57.7)	63 (44.6, 83)	33 (19.4, 43.8)	8 (6.2,9)
Salivary CRP (ng/mL)	1.9 (0.4, 20.6)	1.98 (0.54, 2.95)	0.59 (0.33, 1.44)	0.25 (0.13,0.3)

All values in median (IQR). Group I-Blood culture +/ sepsis screen +; Group II-Blood culture/sepsis screen +; Group III-Clinical/perinatal risk factors only.

WHAT THIS STUDY ADDS?

- Salivary CRP could be used as an alternative biomarker for neonatal sepsis as it increases in septic neonates and positively correlates with serum CRP.

elevated serum CRP, compared to those with only suspicion of sepsis but with non-elevated biomarkers (group 3). We also found a moderate positive correlation of salivary CRP with serum CRP in these neonates.

There is limited previous data on the diagnostic utility of salivary CRP in neonatal inflammatory conditions and its comparison with serum CRP. Iyengar, et al. [9] showed that salivary CRP was detected in 97% of neonates with inflammatory states, especially post-operative. The levels also moderately correlated with serum CRP levels. Omran, et al. [10] found a significant difference between salivary CRP values in septic and healthy infants, with a moderate correlation between salivary and serum CRP. The levels of salivary CRP in our study was different from previous reports [9,10]. Some of the observed differences may be explained by variation in population characteristics. Our study population included both preterm and term infants, whereas others had included mainly post-operative neonates (only 12 with sepsis) [9], or had recruited only term infants [10]. Another reason could be the use of different assay methods and pre-processing techniques. We used a highly sensitive salivary CRP assay [8] and a validated saliva collection method [7]. A significant positive correlation between salivary and serum CRP was also seen by these authors [9,10]. Both adult and pediatric population studies have shown a good correlation between salivary CRP and serum CRP levels, in a variety of clinical conditions [11]. Although positive correlation is reassuring, this is insufficient to advocate salivary CRP as a replacement for serum CRP, considering the population characteristics of our study. More studies in neonates, across a variety of inflammatory conditions, showing similar correlation, are required, in order to change practice.

Proper collection of saliva is important for ensuring accuracy using salivary diagnostics and is even more challenging in neonates. Previous researchers [9,10] had used an improvised 1 mL syringe attached to low-wall suction, to collect saliva, a method previously described by Dietz, et al. [12]. We found this method difficult to use in very low birth weight infants as it frequently resulted in blood mixed saliva, related to mucosal trauma. Hence, we used the SIS for saliva collection, which has been previously validated for salivary analytes [7]. We found that by using a proper technique, maximal uncontami-nated saliva recovery was possible with less patient discomfort.

We found that infants with negative sepsis screen/ blood culture, with only clinical signs or perinatal risk factors of sepsis also had detectable salivary CRP levels (range of 0.11-1.39 ng/mL). This possibly indicates a normal physiological increase in salivary CRP levels to detectable range, in the initial days of life, similar to serum CRP. To our knowledge, there is no previous published normative data on salivary CRP levels in healthy infants. Extrapolation from adult studies could also be fallacious due to the differing population characteristics, and variation in salivary CRP levels among studies, with reported levels ranging from 0.03-24.2 ng/mL [13,14]. Further large studies are necessary to identify normative ranges of salivary CRP in healthy neonates of different gestational ages. The performance of salivary CRP during serial analysis, and also against the 'gold standard' blood culture, was not done in our study. Also, we did not normalize salivary CRP concentration for salivary flow rate and protein concentration, because previous data on the utility of this and the ideal normalization method in neonates was lacking. These areas should also be addressed in future trials.

Our study suggests that salivary CRP could be used as an alternative biomarker to serum CRP in neonatal sepsis. However, widespread usage in neonates will require further research into saliva collection methodology, standardization of assay procedure, establishing normative values and determining cost effectiveness. As of now, it appears a very useful diagnostic surrogate for blood sampling.

Ethics clearance: Chettinad Academy of Research and Education Institutional Human Ethics Committee; No. 121/25/09/2015, dated September 04, 2015.
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