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Sensitivity of miRNA-181a, miRNA-23b and miRNA-16 in the Late-Onset Neonatal Sepsis: A Diagnostic Study

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Abstract

Objective: To evaluate the expression and diagnostic value of levels of the microRNAs (miRNAs), miRNA-181a, miRNA-23b, and miRNA-16, in late-onset neonatal sepsis (LOS) and compare them with the diagnostic utility of C-reactive protein (CRP) levels.

Methods: This was a prospective diagnostic study conducted between January 2021 and March 2023 at a tertiary care center (Sri Ramachandra Hospital) in India. Quantitative real-time polymerase chain reaction was performed to determine miRNA-181a, miRNA-23b, and miRNA-16 expression levels, and CRP was measured by nephelometry. The diagnostic value of miRNA and CRP levels were analyzed using receiver operating characteristic (ROC) curves. ROC curves were utilized to determine optimal cutoff points, and Mann–Whitney tests were performed using SPSS to ascertain P values, with statistical significance defined as <0.05.

Results: This study included 100 samples, with 50 cases of culture-proven LOS (27 females, 23 males) and 50 healthy controls (31 females, 19 males). In LOS, miRNA-181a and miRNA-23b expression levels were significantly downregulated ($P < 0.001$), with area under the curve (AUC) values of 0.83 and 0.92, respectively, whereas those of miRNA-16 were significantly upregulated ($P < 0.001$; AUC = 0.97). In comparison, CRP levels had an AUC value of 0.831 ($P < 0.001$). Further, miRNA-23b showed the highest sensitivity (98%) of markers tested, whereas miRNA-16 exhibited the highest specificity (96%).

Conclusion: MiRNA, especially miRNA-16, shows diagnostic potential for neonatal sepsis compared with traditional biomarkers like CRP and procalcitonin, suggesting its use as an early marker for LOS. However, further cohort studies are needed before practical application.

Keywords: MicroRNAs; Real-time PCR; Blood culture; Biomarkers; Late-onset sepsis

Introduction

Sepsis is a clinical condition induced by invasive infection and characterized by systemic inflammatory response syndrome.[1] The incidence of sepsis in developed countries is 1–8 per 1,000 newborns, whereas rates are 3 times higher in developing countries.[2] Approximately 1 million neonates die annually worldwide due to neonatal sepsis, which may be attributable to the inadequacy of diagnostic tools.[3] Currently, the principal method and gold standard for diagnosing sepsis is blood culture; however, the procedure takes a minimum of 24–48 hours to provide results and has only 60% sensitivity.[4] To overcome these difficulties, several predictors of neonatal sepsis have been identified, including procalcitonin (PCT), C-reactive protein (CRP), microerythrocyte sedimentation rate, and interleukin (IL) levels[5]; however, all of these parameters are general indicators of inflammation, which lack either sensitivity or specificity. Hence, improved early identification of neonatal sepsis is a priority aim for modern medicine. Regardless of our best efforts, neonatal sepsis remains a global burden and a major source of mortality, organ dysfunction, and high hospitalization costs.[6] As early diagnosis of sepsis is crucial for improving survival rates among neonates, identifying ideal biomarkers for its diagnosis and prognosis is an urgent priority.[7] Hence, researchers continue to search for rapid and accurate biomarkers, with high sensitivity and specificity, to detect and eliminate sepsis at the earliest possible opportunity.[8]

MicroRNAs (miRNAs) are non–protein-coding RNA molecules of 18–25 nucleotides that are associated with posttranscriptional gene regulation. The roles of miRNAs are vital in several biological processes, including cell proliferation, metabolism, and many other physiological activities. Circulating miRNAs can serve as biomarkers for diagnosing various infections.[9] Vasilescu *et al.*[10] first identified miRNA-150 in plasma as a probable indicator of sepsis. Since then, numerous miRNA-related studies have emerged, verifying the significance of these molecules in sepsis. MiRNA-23b plays an anti-inflammatory role by targeting matrix metalloproteinase 10 and negatively regulates responses induced by

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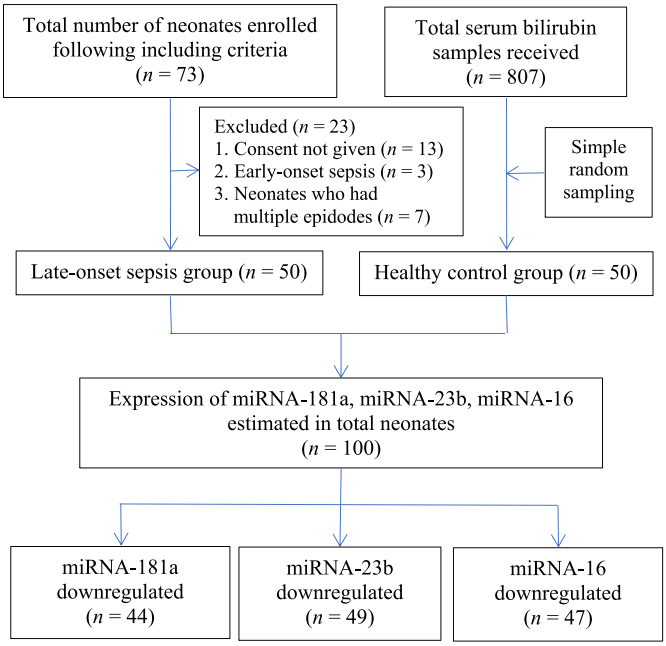

Figure 1. The STARD diagram of the participants' flow through the study. STARD: Standards for reporting of diagnostic accuracy studies.

lipopolysaccharide, and its downregulation is an indicator of sepsis development.[11] He *et al.*[12] demonstrated that miRNA-181a elevates immune thrombocytopenia by regulating toll-like receptor 4 (TLR-4), a key factor in the innate immune system, leading to the development of neonatal sepsis. Further, Chen *et al.*[13] identified downregulated miRNA-181a expression in patients with neonatal sepsis. The role of miRNA-16 in the innate immune system involves downregulation of the transcription of TLR-4 and IL-1 receptor–associated kinase 1, whereas Wang *et al.*[14] found that miRNA-16 expression was significantly upregulated in patients with sepsis.

In this study, we focused on assessing the expression levels of miRNA-181a, miRNA-23b, and miRNA-16 in newborns diagnosed with culture-proven late-onset sepsis (LOS) relative to those in healthy controls. In addition, we determined the diagnostic value of these miRNAs for detecting culture-positive neonatal sepsis. As a secondary objective, we compared expression levels of these miRNAs between survival and nonsurvival groups of patients with LOS.

Materials and methods

Study design and sample collection

This was a prospective diagnostic study conducted from January 2021 to March 2023 at Sri Ramachandra Hospital, which is a tertiary care center in India. Data collection was planned before the index tests, and reference standards were analyzed. Samples from neonates, both term and preterm (>25 weeks gestation), admitted to neonatal intensive care unit after 48 hours of life with signs and symptoms of sepsis and culture-proven LOS, were enrolled in this study as the sepsis group. Neonates with early-onset sepsis (EOS) within 48 hours were excluded (Fig. 1).

Serum bilirubin samples from neonates in the postnatal ward with no clinical or laboratory signs of sepsis were included as the healthy control group. Among samples received and deemed eligible (n = 880) during the study period, a simple random sampling method was followed. Of 73 confirmed cases of LOS, 50 samples were included in the sepsis group, whereas 50 of 807 serum bilirubin samples were included in the control group. The levels of miRNA were assessed as index markers for their diagnostic accuracy. Assay values were compared with those of the gold standard, culture positivity, and the routine sepsis marker, CRP, and their sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) evaluated.

C-reactive protein

D-Serum samples were sent for analysis on the same day as cultures were measured for CRP levels using the nephelometric method. Nephelometry is a methodology used to quantify the amount of light scattered by CRP particles in patients' blood samples. Data on culture-proven LOS, CRP, and numbers of samples in survival and nonsurvival groups were documented prospectively.

RNA extraction and miRNA enrichment

All serum samples were thoroughly thawed and centrifuged at 700 × g for 10 minutes to remove cell debris. Then, 100 µL serum sample aliquots were transferred into 2 mL DNA-LoBind tubes, and 50 µL denaturing buffer (Sigma Aldrich, Burlington, MA) was added, according to the protocol (miRNeasy kit (Qiagen, Hilden, Germany)). The mixture was then centrifuged at 1000 × g, gently mixed every 15 seconds, and then left at room temperature to inactivate serum RNAase. Then, 20 fmol of synthetic miRNA oligonucleotides from Integrated DNA Technologies (Coralville, Iowa) was added.

Total RNA was extracted from serum samples using a miRNeasy kit (Qiagen) following the manufacturer's guidelines. Extracted RNA was purified for miRNA enrichment using a miRVana PARIS kit (Ambion, Austin, TX), following the manufacturer's guidelines. Further RNA elution and purification were then conducted (Norgen Biotek, Thorold, ON, Canada), miRNA eluted in 50 µL nuclease-free water

Table 1

Comparison of miRNA-181a, miRNA-23b, and miRNA-16 with different culture positivity.

	miRNA-181a (downregulated)		miRNA-23b (downregulated)		miRNA-16 (upregulated)	
Culture (n)	Yes	No	Yes	No	Yes	No
Gram-negative (38)	32	6	37	0	34	4
Gram-positive (10)	10	0	10	0	7	3
Fungi (2)	2	0	2	0	2	0
Total (50)	44	6	49	1	43	7

Table 2

Comparison of miRNA-181a, miRNA-23b, and miRNA-16 between the study groups (*n* = 100).

Study group (*n*)	miRNA-181a Median (IQR)	*P*	miRNA-23b Median (IQR)	*P*	miRNA-16 Median (IQR)	*P*
Sepsis group (50)	0.38 (0.28, 0.53)	<0.001	0.16 (0.08, 0.25)	<0.001	3.08 (2.55, 3.95)	<0.001
Control group (50)	2.73 (0.99, 3.27)		2.23 (1.04, 3.62)		0.47 (0.33, 1.18)	
Survival group (44)*	0.38 (0.28, 0.55)	0.89	0.16 (0.08, 0.25)	0.76	3.08 (2.53, 3.96)	0.69
Nonsurvival group (6)*	0.38 (0.37, 0.48)		0.12 (0.06, 0.21)		2.98 (2.58, 3.65)	

*Total number of neonates in survival group were 44 and in non survival group were 6. The neonates were retrieved from sepsis group, who had confirmed blood culture reports and admitted in neonatal intensive care unit with signs and symptoms of sepsis.

IQR: interquartile range.

(Ambion), and the concentration was estimated using a Nanodrop instrument.

Reverse transcription

To synthesize complementary DNA for miRNA profiling, synthetic miRNAs, namely, miRNA-23b, miRNA-16, miRNA-181, and miRNA-U6 (Internal control), were reverse transcribed using commercially available primers and a TaqMan miRNA reverse-transcription kit (Applied Biosystems, Foster City, CA). Reactions were carried out using 5 µL RNA in a reaction volume of 15 µL.

Quantitative real-time polymerase chain reaction

To quantify the number of miRNAs, real-time polymerase chain reaction (PCR) assays were conducted using TaqMan miRNA probes and TaqMan Master Mix without Amp Ease UNG (Applied Biosystems), following the guidelines provided by the manufacturer. This allowed accurate measurement of both synthetic miRNA spike-ins and cellular miRNAs. The miRCURY LNA human miRNA real-time PCR panel I and miRCURY SYBR Green Master Mix were used for miRNA profiling, following the kit guidelines. The specific miRNAs targeted for analysis included miRNA-23b, miRNA-16, miRNA-181, and miRNA-U6. Complementary DNA was diluted 55-fold for use in a 384-well PCR panel analysis. ROX reference dye (1 µL) was added to the master mix, and real-time PCR was conducted in a total volume of 10 µL using an ABI 7900HT real-time PCR system (Applied Biosystems).

PCR assays were carried out in duplicate, and raw cycle threshold (Ct) values for each sample were determined as the mean of 2 individual Ct values. The U6 housekeeping gene was simultaneously amplified to standardize the quantity of miRNA in each sample. Gene transcription levels were quantified using the delta-delta (CT $2^{-\Delta\Delta CT}$) method.

Statistical analysis

Categorical data are presented as either numbers or percentages. Numerical data are expressed as mean ± standard deviation if normally distributed, or median and interquartile range if non–normally distributed. Receiver operating characteristic (ROC) curves were constructed to derive cutoff points with the highest sensitivity, specificity, PPV, and NPV. The Mann–Whitney *U* test was conducted, and *P* values derived using Statistical Package of Social Sciences software, with statistical significance set as <0.05.

Ethical approval

This study was approved by the Institutional Ethical Committee (IEC-N1/21/FEB/77/40), and informed consent was acquired from any one of the parents of all neonates enrolled. All methods were carried out in accordance with the ethical standards of the ethics board and conformed to the *Declaration of Helsinki*.

Results

Characteristics of late-onset sepsis samples

A total of 100 samples were included in this study: 50 from patients with culture-proven LOS (27 (54%) female and 23 (46%) male) and 50 from healthy controls (31 (62%) female and 19 (38%) male). Of 50 positive cultures, 38 (76%) were gram-negative, including 17, 9, 5, 3, 2, 1, and 1 *Klebsiella pneumoniae* isolates, *Acinetobacter*, *Escherichia coli*, *Enterobacter*, *Pseudomonas*, *Elizabethkingia meningoseptica*, and *Ralstonia mannitolilytica* isolates, respectively. Further, 10 (20%) positive cultures were gram-positive isolates including 5 coagulase-negative *Staphylococcus aureus* and 5 *Enterococcus* isolates, whereas 2 (4%) were *Candida auris* fungal isolates. MiRNA-181a and miRNA-23b were significantly downregulated, whereas miRNA-16 was significantly upregulated in samples positive for these isolates relative to levels in healthy controls (Table 1). In these 50 neonates with culture-proven sepsis, 33 had apnea, 3 had jaundice, 3 had seizures/abnormal movements, 19

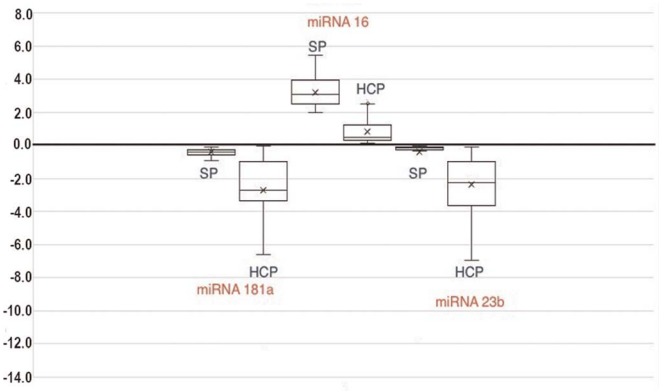

Figure 2. Expression levels of miRNA181a, miRNA-23b and miRNA-16 compared between sepsis group and control group. Y-axis is $2^{\Delta\Delta CT}$. SP: sepsis; HCP: healthy control group.

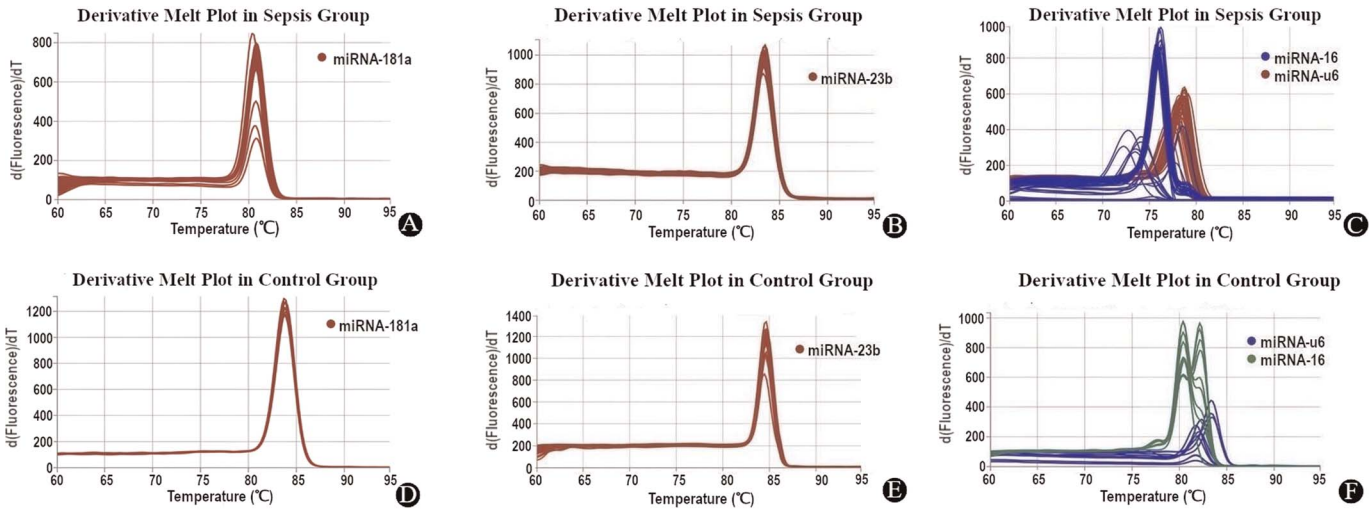

Figure 3. Derivative melting curve of miRNA-181a, miRNA-23b, and miRNA-16 in the control and sepsis groups. A Derivative melt plot in sepsis group in miRNA-181a. B Derivative melt plot in sepsis group in miRNA-23b. C Derivative melt plot in sepsis group in miRNA-16 and miRNA-u6. D Derivative melt plot in control group in miRNA-181a. E Derivative melt plot in control group in miRNA-23b. F Derivative melt plot in control group in miRNA-16 and miRNA-u6. d(Fluorescence)/dT means change in fluorescence level with respect to per unit change (increase or decrease) in temperature (°C).

had bradycardia/tachycardia, 7 had hypo/hypertension, 30 had a central line, 3 had arterial blood gas abnormalities, 1 had lethargy, 3 had abdominal distension, 2 had shock, 43 were on respiratory support, and 6 neonates died.

miRNA-181a, miRNA-23b, and miRNA-16 expression levels

Levels of miRNA-181a, miRNA-23b, and miRNA-16 expression were assessed by quantitative real-time PCR and compared between the sepsis and control groups (Table 2). Levels of miRNA-181a and miRNA-23b expression were significantly lower in patients with sepsis than those in the control group ($P < 0.001$), whereas levels of miRNA-16 were significantly higher in LOS cases than those in the control group ($P < 0.001$) (Fig. 2).

MiRNA melting curve plots are presented in Figure 3. The standards showed a gradual increase in threshold (C_t) value with decreasing miRNA concentration. To determine the melting temperatures of the miRNAs, melt curve analyses were conducted at 65°C to 95°C. The melting temperatures for miRNA-181a, miRNA-23b, and miRNA-16 were 83° C ± 2°C, 83°C ± 2°C, and 78°C ± 2°C, respectively.

Diagnostic value of miRNA-181a, miRNA-23b, miRNA-16, and C-reactive protein

To assess the diagnostic value of miRNA-181a, miRNA-23b, miRNA-16, and CRP levels for discriminating LOS from healthy controls, we conducted an ROC curve analysis. We plotted the results of the gold standard method, blood culture, against $2^{-\Delta\Delta CT}$ (fold-change) values of individual miRNA expression levels and CRP levels to generate ROC curves (Fig. 4, Table 3). Cutoff points associated with the highest sensitivity and specificity values were derived from these ROC curves. For miRNA-181a, a cutoff value

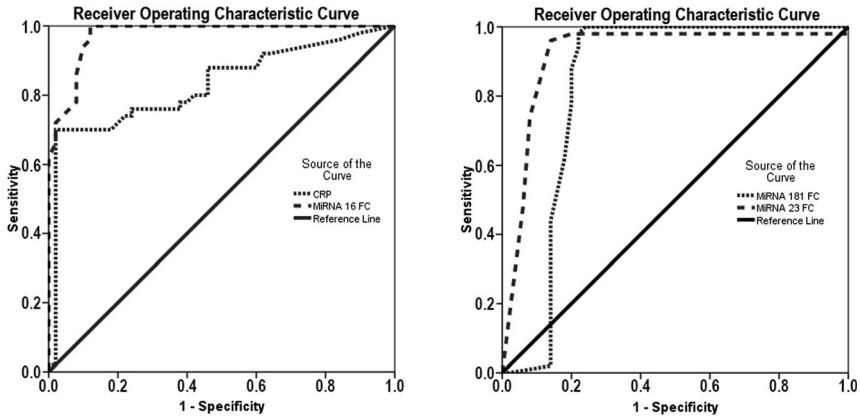

Figure 4. Analysis of the receiver operating characteristic curve for miRNA-181a, miRNA-23b, and miRNA-16. Diagonal segments are produced by ties. CRP: C-reactive protein.

Table 3

Area under the curve of miRNA-181a, miRNA-23b, miRNA-16 and C-reactive protein (*n* = 100).

Test result variable	Area under curve	95% confidence interval	*P*
miRNA-181a	0.834	0.737–0.931	0.001
miRNA-23b	0.920	0.856–0.984	0.001
miRNA-16	0.975	0.950–0.999	0.001
CRP	0.831	0.746–0.915	0.001

CRP: C-reactive protein.

of 0.65 relative units corresponded with a sensitivity of 88%, specificity of 80%, PPV of 81%, NPV of 86%, and AUC of 0.834. For miRNA-23b, a cutoff value of 0.55 relative units corresponded with a sensitivity of 98%, specificity of 80%, PPV of 83%, NPV of 97%, and an AUC of 0.920. Further, a cutoff value of 2.25 relative units for miRNA-16 corresponded with sensitivity of 86%, specificity of 92%, PPV of 91%, NPV of 86%, and AUC of 0.975. In comparison, a CRP cutoff value of 3.85 mg/L corresponded to sensitivity of 74%, specificity of 78%, PPV of 77%, NPV of 75%, and AUC of 0.831. Figure 5 shows a heat map of the differential expression of miRNA-181a, miRNA-23b, and miRNA-16.

Discussion

In the present study, we analyzed the expression levels and clinical significance of 3 candidate miRNAs (miRNA-181a, miRNA-23b, and miRNA-16) in neonatal patients with LOS. Quantitative reverse transcriptase–PCR analysis of serum from neonates revealed differential expression of these miRNAs between patients with LOS and healthy controls. Levels of both miRNA-181a and miRNA-23b expression in serum from neonatal patients with LOS were identified as significantly lower than those in healthy controls, whereas levels of miRNA-16 were significantly higher in neonatal patients with sepsis. A similar study by Liu *et al*., where miRNA-181a expression levels were compared in 102 patients with neonatal sepsis (NS) and 50 controls, reported significant downregulation of miRNA-181a in patients with sepsis relative to those in controls.[3] Another similar study by Chen *et al*. reported a downregulation of circulating miRNA-181a in patients with NS,[13] as well as a 2-fold downregulation of miRNA-23b in patients with NS. Further, Fatmi *et al*. demonstrated that the miRNA-23b expression levels in 48 enrolled neonates could be used to divide patients into groups with EOS (*n* = 27) and LOS (*n* = 21). They reported significant upregulation of miRNA-23b in EOS and significant downregulation in the LOS group.[15] Moreover, Ou *et al*. compared miRNA-23b levels in 87 patients with NS and 50 controls, as well as between 16 nonsurvivors and 71 survivors, and found that they were significantly lower in both the NS and nonsurvival groups.[16] In this study, we found that miRNA-16 was significantly upregulated by more than 2-fold in patients with NS. Wang *et al*. reported significant upregulation of miRNA-15a/16 3-fold in 46 patients with NS relative to levels in 41 control patients.[2] Finally, El Hamshary *et al*. reported significantly higher levels of miRNA-16 in 40 patients with NS than those in 20 controls.[17]

In this study evaluating the clinical significance of miRNA-181a, miRNA-23b, and miRNA-16 in neonatal LOS, we demonstrate that these markers exhibit both sensitivity and specificity values >80%. To derive the cutoff values for these miRNAs for diagnosis, we conducted ROC analysis. For miRNA-181a, a cutoff value of 0.65 relative units corresponded with a sensitivity of 88%, specificity of 80%, and AUC of 0.834, consistent with the findings of Liu *et al*., who found that miRNA-181a has a significant value for sepsis diagnosis, with an AUC value of 0.893 at a cutoff value of 0.625 (sensitivity and specificity, 83% and 84%, respectively).[3] Further, we found that a cutoff value of 0.55 relative units for miRNA-23b showed the highest sensitivity (98%), with a specificity of 80% and AUC of 0.920, whereas an miRNA-16 cutoff value of 2.25 relative units corresponded to a sensitivity of 86% and the highest specificity (92%), with AUC of 0.975. This result agrees with the report of El Hamshary *et al*., who found that miRNA-16 has a significant value for the diagnosis of sepsis at a cutoff value of 353.6 (sensitivity 82%, specificity 70%, and AUC 0.802).[17] This cutoff varies from the cutoff we provided in this study. This might be due to different methodologies and different kits employed in respective studies. Our results are also consistent with those of Wang *et al*., who found that the AUC of miRNA-16 was 0.8688.[14]

There remains a place for analysis of CRP and PCT in the diagnosis and prediction of prognosis of patients with sepsis in the clinic, due to their ease of analysis. In our study, CRP yielded an AUC value of 0.831. Nevertheless, although

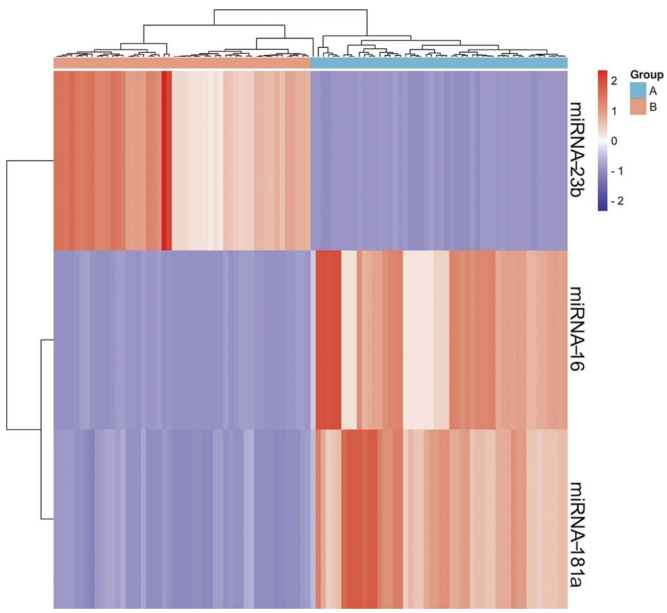

Figure 5. Heat map visualization of the normalized microRNA expression levels for the control (A) *vs*. sepsis (B) sample. An SRplot was used to investigate the bidirectional gene clusters and a heat map was generated to gain deeper understanding of the specified microRNA expression of miRNA-23b, miRNA-16, miRNA-181a in the control *vs*. sepsis sample (*n* = 100).

these are established markers, their AUC values are generally in the range of 0.83–0.87,[18] whereas in this study, the AUC values for all 3 miRNAs were >0.80. In particular, miRNA-16 had the highest AUC (0.97), which is considerably higher than those of the routinely available biomarkers, CRP and PCT, demonstrating that miRNA-16 has high diagnostic accuracy for LOS, which is a strength of this study; however, we did not collect samples at different time intervals, representing a limitation of our investigation. Further, the sample sizes of the survival and nonsurvival groups were too small, which is another potential limitation of this study.

Among culture-proven sepsis samples, 90% showed a downregulation of miRNA-181a and miRNA-23b. Similarly, all culture-proven samples showed upregulation of miRNA-16. Eighty-four percent of healthy controls were negative, demonstrating that miRNA can serve as a marker of neonatal LOS. Because miRNA can be analyzed by PCR, with a typical turnaround time of 5 to 6 hours, it can be used as an early indicator of neonatal sepsis. Hence, further investigations with large sample cohorts are required to evaluate the changes in these miRNA molecules during sepsis.

In this study, we demonstrated the role of miRNA181a, miRNA-23b, miRNA-16, and CRP in the late onset of neonatal sepsis. The expression levels of miRNA181a and miRNA-23b were significantly downregulated, and the expression levels of miRNA-16 were significantly upregulated in LOS. MiRNA-23b showed a high sensitivity of 98%, and miRNA-16 showed a high specificity of 92% and the highest diagnostic accuracy of AUC 0.97 than any other routinely available sepsis markers.

Conclusion

MiRNA levels show promising diagnostic potential for neonatal sepsis, with miRNA-16 demonstrating a superior AUC compared with traditional biomarkers like CRP and PCT, suggesting their utility as early markers for LOS. MiRNA holds great promise for sepsis diagnosis. However, extensive cohort studies are necessary before these biomarkers can be practically applied.

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Author Contributions

Divya Katta: principal investigator. K.S. Sridharan: Conceptualization, design, and manuscript preparation. Umamaheswari Balakrishnan, Prakash Amboiram, Kennedy Kumar: clinical data collection, analysis, and proofreading. All authors contributed to data analysis and interpretation, reviewed, and approved the final manuscript.

Conflicts of Interest

None.

Data Availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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