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Biochemical and molecular study on serum miRNA-16a and miRNA- 451 as neonatal sepsis biomarkers

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ABSTRACT

Background: Sepsis is the serious cause of fatality in the unit of medical-intensive care (ICU). Non-coding RNA transcripts are microRNA that control gene expression by repressing translation or degrading mRNA. There are several reports discussing the concept of using miRNAs as sepsis a biomarkers by profiling miRNA dysregulation in sepsis patients' blood samples.
Objectives: The research was aimed at exploring the clinical utility of miRNA-16a and miRNA- 451 for diagnosis of neonatal sepsis.
Subjects: and methods: This research was conducted on 50 full term neonates, 25 neonates with suspected or proven sepsis and 25 clinically healthy sex and age matched neonates with no evidence of sepsis. All newborns have been exposed to clinical review, history taking and laboratory investigations including total & differential count of blood cells, C-reactive protein, blood culture. Serum miRNA-16a and miRNA-451 levels have been assessed using Real Time polymerase chain reaction (Real Time PCR) technique.
Results: Neonates with sepsis had considerably higher levels of miRNA-16a and miRNA- 451 than the healthy neonates ($p \leq 0.001$). Receiver operating curve (ROC) showed that serum miRNA-16a was superior to miRNA-451 for diagnosis of sepsis with neonatal origin; it had sensitivity and specificity of 88% and 98% versus 64% and 61% respectively. Cut off point for miRNA-16a to diagnose neonatal sepsis was above or equal 3.16. Also, cut off point for miRNA-451 was above or equal 1.26. miRNA-16 a and miRNA 451 expression was significantly correlated with respiratory rate, WBCs, and CRP.
Conclusion: Both miRNA −16a and miRNA-451 are detected in higher levels in newborn with sepsis compared to controls. MiRNA- 16a could be considered as promising biomarkers for diagnosis of neonatal sepsis.

1. Introduction

Neonatal infections, particularly sepsis of neonates, continue to be a worldwide cause of substantial mortality and morbidity. It is described as infection of blood mainly triggered by bacteria that occur within the first 28 days after birth, the most common pathogenic agents causing sepsis of neonates are group B streptococci (GBS), *Escherichia coli, Haemophilus influenza, Coagulase-negative Staphylococcus and Listeria monocytogenes* [1] Its incidence is approximately three times in developing countries rather than in developed one [2]. The organization of world Health (WHO) reports that a million fatalities every year (10% of all those under five mortality) are owed to sepsis of neonates, as well as 42% of such deaths take place in first week after birth [3]. Microbiological culture in the distinctiveness of sepsis from other non-infectious diseases is the standard of gold; however, this technique takes time and frequently connected to fake negative results [4]. The neonatal sepsis diagnosis also poses an extremely false adverse rate and a delay in the acquisition of results from blood culture due to the nonspecific clinical symptoms. Thus, there is an urgent need for biomarkers to reliably distinguish sepsis at an earlier level [1]. MicroRNAs are series of short,

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retained non-coding RNAs nucleotide lengths of 20–25 the ones who are correlated in the cell with different, pathological, physiological, and important processes. The most of microRNAs participate in the process of directing the complex silencing caused by RNA (RISC) for targeting modulate expression post-transcriptionally and mRNAs via binding with the RISC to the 3′ un-translated area (UTR) of the mRNA [5]. We aimed at investigating the serum miRNA-16a and miRNA- 451 as biomarkers for neonatal sepsis.

2. Subjects and methods

2.1. Subjects

This research was conducted at Biochemistry department, Science Faculty in cooperation with Medical Biochemistry and Molecular Biology, Microbiology and Immunology and Intensive Care Unit of neonates of Pediatric Department, Faculty of Medicine, Menoufia University, Egypt in the period from February 2019 to August 2020. It was performed on 50 neonates, divided into two classes: **group I** comprised of 25 neonates with sepsis, they were diagnosed by sepsis signs and symptoms in accordance to clinical sepsis score: (observation range 0–4.5 = no sepsis, >5–10 = sepsis), and **group II** comprised of 25 clinically healthy newborns which are full term with no evidence of neonatal sepsis; matched for sex and age with the patients group before discharge from NICU or at follow up visit, they served as controls. The exclusion criteria were: congenital infection, suspected inborn metabolism errors, perinatal asphyxia, congenital defects, chromosomal disorders, preterm neonates and infant of diabetic mother.

Ethical approval

Before collection of sample, written informed consent (Accepted by the Rights of Human and Ethics Committee in Research of Medicine Faculty, Menoufia University) has been obtained from all of the mothers.

A complete history had been taken, and thorough clinical examination and laboratory investigations included routine investigations: total and differential count of blood, C-reactive protein and culture of blood. Assessment of serum miRNA-16- a and miRNA- 451 levels with use of real time PCR technique, performed for all subjects.

2.2. Sampling and primary test

2 ml of blood samples have been gathered in no additive tubes from 25 neonates with sepsis and 25 healthy neonates and centrifuged at 4000 rpm for 10 min at room temperature to separate cell debris, and then the supernatants had been stored at −80 °C till the extraction of RNA**(1)**.

2.3. Micro RNA 16a and 451 expressions by real-time PCR

MiRNA extraction from 100 μl of serum samples; total RNA including miRNA were extracted using miRNeasy isolation kit (QIAGEN, USA). RNA concentration and quality had been evaluated using of Nano Drop tool (Thermo Scientific, USA), RNA extract was stored at – 80 °c. Reverse transcription was conducted using the kit of miScript II RT (QIAGEN, USA) for production of complementary DNA (cDNA). Each reaction had been performed on ice with a volume of 20 μl totally as follows, RT Master Mix was prepared: 4 μl miScript HiSpec RT buffer +2 μl miScript Nucleic Mix +2 μl miScript ™ Transcriptase reverse +2 μl Nuclease- Free Water have been pipetted into each well. 10 μl of miRNA extract was pipetted into each well. Incubation was performed with use of thermal 2720 cycler Singapore, Applied Bio system for single cycle as the following: 37 °c for 60 min, 95 °c for 5 min to inactivate reverse transcriptase. cDNA had been deposited at – 20 °c till stage for real time PCR. Real-time PCR was performed using PCR Kit miScript SYBR Green (QIAGEN, USA), prior to amplification, cDNA samples had been diluted with Nuclease- Free water in a ratio with 1:5 (4 μl cDNA + 16 μl of water), a volume of 25 μl totally was applied, in the form of 12.5 μl of SYBR green Master Mix; 3.5 μl of Nuclease-free water, 4 μl of diluted cDNA and 2.5 μl of miScript universal primer, 2.5 μl of miScript primer assay. The small nuclear U6 was used for normalization. The following primers had been used; mature miRNA −16a CCAGUAUUAACUGUGCUGCUGA and miR-451 AAACCGUUACCAUUACUGAGUU and mature U6 as a house keeping gene (miScript primer assay kit, QIAGEN, USA). The data was analyzed with the aid of ABI7500 real-time PCR instrument with software V.2.0.1, ABI7500 including the conditions below for cycling: initial phase of activation was done at 95 °c for 15 min, accompanied by three step cycling for 40 cycles, (denaturation) 94 °c for 15 s then (primer annealing) 55 °c for 30 s, then (primer extension) 70 °c for 30 s. Relative quantification (RQ) levels of expressions were calculated with aid of the comparative $2^{-\Delta\Delta Ct}$ method with Applied Bio system 7500 software version 2.0.1. Where the amount of wanted miRNA-16a and miRNA-451 had been normalized to an endogenous reference U6 small RNA and relative to control. Each run was completed using analysis of melting curves (Fig. 1, Fig. 2) to confirm the particularity of the amplification (Fig. 3) and absence of primer dimers.

2.4. Statistical analysis of the data

Data had been evaluated with aid of the IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp.). The qualitative data was shown as percent and numbers. The normality of the distribution had been analyzed by the Kolmogorov-Smirnov test. Means, typical deviations, and medians had been used to the quantitative data. The findings were considered to be significant at p value less than 0.05%.

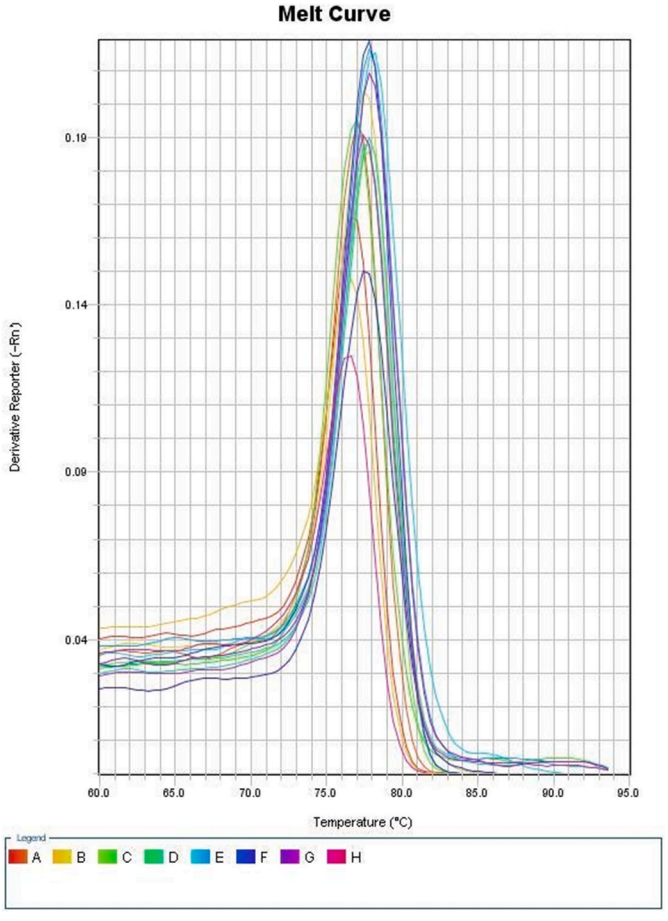

Fig. 1. Melting curve to ensure the specificity of selected primers for miRNA-16a.

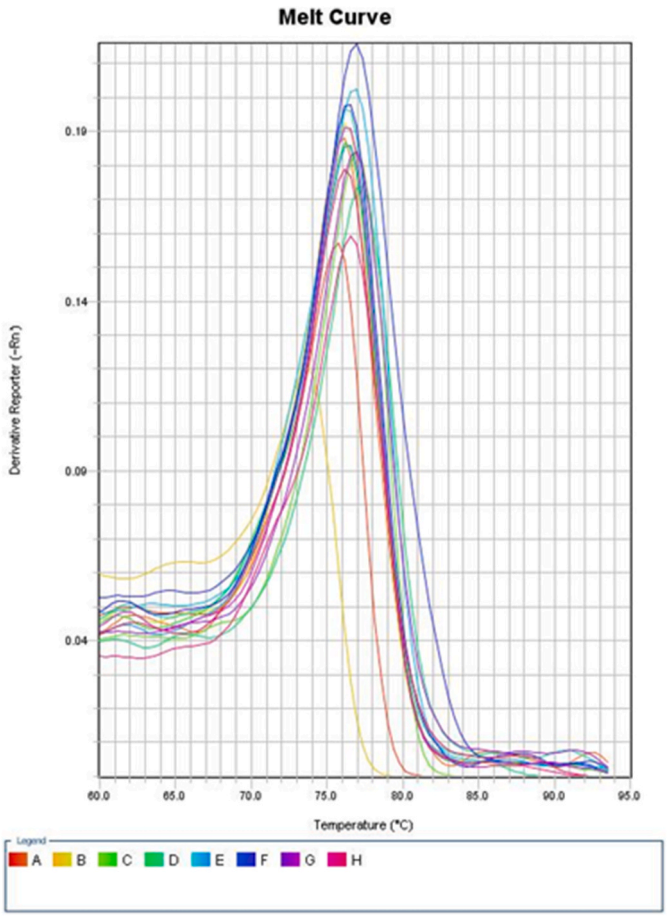

Fig. 2. Melting curve to ensure the specificity of selected primers for miRNA-451.

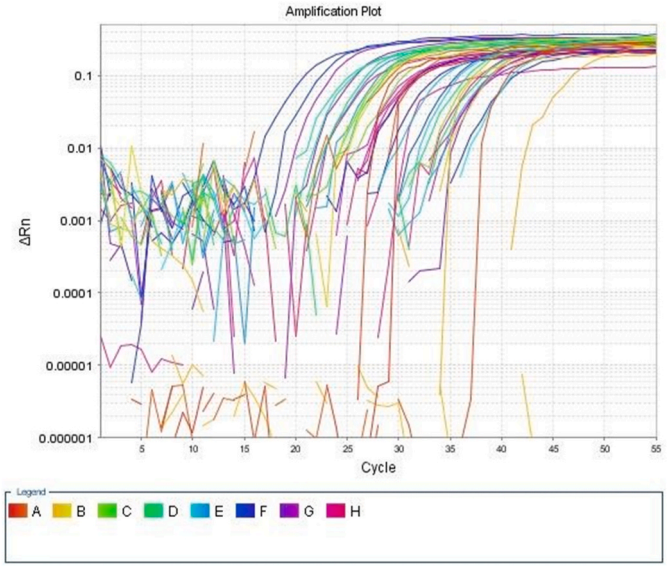

Fig. 3. Amplification plot of both miRNA- 16a and miRNA-451 (normalized fluorescence signal (Rn)) plotted versus cycle number.

3. Results

In this research: by comparison between the two studied groups (cases and controls) according to personal history, the average age of patients was 1.72 ± 2.11 days; the most of them were females (52%). Also, the bulk of the patients (72%) was the first baby, but with no discrepancy which is statistically important between both groups (Table 1). The comparison between the two studied categories according to antenatal history and family history showed that the gap between the two groups was not statistically important, however by comparing the two categories as regarding natal history, there was highly significant association between birth resuscitation and neonatal sepsis ($p = 0.001$). On the other side, no noticeable differences between septic cases and controls regarding the mode of delivery ($p = 1$), complications of delivery ($p = 1$), and gestational age ($p = 0.94$) **(Data not shown).** Laboratory investigations revealed that sepsis patients had significantly lower count of platelets than normal neonates. On the other side, septic cases had statistically significant higher CRP levels and positive cultures of blood in 92% of cases while blood cultures were negative in all normal neonates (100%) with highly significant statistical discrepancy between the categories ($p \leq 0.001$), the most commonly encountered organisms in the current study was respiratory tract pathogens, followed by gastrointestinal pathogens (Table 2). Sepsis patients had substantially greater level of miRNA-16a than the healthy neonates ($p \leq 0.001$) (Table 3), Figure (4A). Also, there was difference that is statistically important observed between both categories as regards miR-451 ($p = 0.034$), with greater level of miRNA-451 in septic babies than the healthy ones (Table 4), Figure (4B). The ROC curve for various parameters to estimate cases (vs. controls) stated that the serum miRNA-16a was superior to miRNA-451 for sepsis diagnosis with sensitivity and specificity of 88% and 98% versus 64% and 61% respectively. Cut off point for miRNA-16a for diagnosis of neonatal sepsis was equal or above 3.16. Also, cut off point for miRNA-451 was equal or above 1.26 (Table 5), Figure (5). In our research, miRNA-16a expression correlated significantly with respiratory rate ($r = 0.63$; $p = 0.001$), WBCs count ($r = -0.62$, $p = 0.001$), and CRP levels ($r = 0.448$, $p = 0.025$). Likewise, miRNA 451 expression correlated significantly with respiratory rate ($r = -0.415p = 0.039$), WBCs count ($r = -0.44$, $p = 0.027$), and CRP levels ($r = -0.459$, $p = 0.021$) (Table 6).

4. Discussion

Owing to non-specific clinical presentations, the neonatal sepsis diagnosis is still considered a concern for both clinicians and laboratory specialists. The value of early diagnosis of sepsis cannot be overestimated because of its high mortality, high rate of failure of organs and high hospital costs. Therefore, fast and accurate biomarker identification of sepsis can save lives, especially if it can be a point-of-care review [6]. Traditional markers for pediatric sepsis include WBC & neutrophils count, CRP and procalcitonin [7]. On the other side, thrombocytopenia is one of sepsis manifestations; the degree of severity of thrombocytopenia is generally proportional to the seriousness of the infectious disease [8]. Thrombocytopenia in sepsis by direct toxic injury to platelets, megakaryocyte suppression, increased consumption from the periphery as in DIC, or the existence of an immune component due to evaluated

Table 1
Comparison between the two studied groups according to personal history.

	Cases (n = 25)		Control (n = 25)		P
	No.	%	No.	%	
Sex					
Male	12	48.0	11	44.0	
Female	13	52.0	14	56.0	0.777
Order in birth					
First	18	72.0	13	52.0	
Second	5	20.0	8	32.0	MCp =
Third	2	8.0	4	16.0	0.405
Age(day)					
Min. –Max	1.0–11.0		1.0–15.0		
Median (IQR)	1.0 (1.0–1.0)		1.0 (1.0–2.0)		0.207

Table 2
Comparison between the two studied groups regarding laboratory data.

	Cases (n = 25)		Control (n = 25)		P
	No	%	No	%	
WBC					
Min- Max	4.0–29.70		5.50–18.40		
Median (IQR)	11.70 (9.40–15.30)		12.40 (9.30–14.50)		0.587
(g/dl) **HB**					
Min. – Max.	8.80–15.60		6.50–18.60		
Median (IQR)	12.40 (10.7–13.80)		15.0 (11.70–16.0)		0.074
PLT					
Min. – Max	27.0–999.0		13.50–928.0		
Median (IQR)	156.0 (62.0–248.0)		275.0 (186.0–335.0)		0.023*
CRP					
Negative	0	0.0	25	100.0	<0.001*
Positive	25	100.0	0	0.0	
Min – Max	12.0–308.20		12.0–308.20		
Median (IQR)	78.40 (28–126.0)		78.40 (28.0–126.0)		–
Blood culture					
No growth	2		25	100.0	
Gram –Ve	22	88.0	0	0.0	MCp
Gram + Ve	1	4.0	0	0.0	<0.001
Bacilli most probably klebsiella	2	8.7	0	0.0	–
Bacilli mostly klebsilla	12	52.2	0	0.0	–
Bacilli most probably enterobacter	4	17.4	0	0.0	–
Most probably enterobacter	1	4.3	0	0.0	–
Bacilli mostly E coli	2	8.7	0	0.0	–
Multidry resistance enterobacter	1	4.3	0	0.0	–
Most probably staphylococcus	1	4.3	0	0.0	–

WBC: white blood cell count, Hb: Hemoglobin, PLT: platelets and CRP: c reactive protein.

Table 3
Comparison between the two studied groups according to miR 16.

MiRNA- 16a	Cases (n = 25)	Control (n = 25)	p
Min. – Max.	0.92–11.88	0.31–3.23	
Median (IQR)	5.75 (3.55–9.15)	0.90 (0.41–1.03)	<0.001*

platelet level -associated immunoglobulins [9]. Our analysis revealed that sepsis patients had significantly lower platelet count. In addition, septic cases had statistically significant higher CRP levels. Similar to our findings: [10-12]. In the diagnosis of sepsis, bacterial culture remains the gold standard; in addition, the findings take at least 24–48 h, having just 60% sensitivity. Biological markers, such as interleukins, CRP, ILs, procalcitonin, and sedimentation levels of erythrocytes (ESR) have high sensitivity but poor specificity. So, the need of the hour is to develop latest methods for diagnosis that are rapid and more specific [13]. In this research, the levels of expression of circulating microRNAs (miRNAs) involved in controlling genes for immune response and underlying inflammatory reactions were estimated, which can be used for diagnose sepsis. MiRNA-16a is a small RNA non-coding molecule situated on chromosome 13q14. There have been claims that MiRNA-16a can significantly inhibit cell proliferation and invasion, promote cell apoptosis and suppress cell cycle progression. It was revealed that transfection with MiRNA-16a mimic suppressed the secretion and mRNA expression of pro-inflammatory factors, such as interleukin 6 and Tumor necrosis factor-alpha (TNF-alpha), whereas it enhanced the secretion and mRNA expression of the anti-inflammatory factor IL-10 [14] On the other side, miRNA-451 gene is located at 17q11.2 on chromosome 17, 100 bp downstream of the miR-144 gene, MiRNA-451 was first identified in the pituitary gland of humans by Ref. [15]. The gene encoding this miRNA participates in multiple pathological and physiological processes, including hematopoietic system differentiation [16]. Previous reports showed that MiRNA-451 were selectively higher in Gram-negative sepsis with *E. coli* [17]. In the current study, we observed that sepsis patients had considerably higher levels of miRNA-16a and miRNA- 451 than the healthy neonates. The ROC curve demonstrated that the serum miRNA-16a was superior to miRNA-451 for diagnosis of the sensitivity and specificity of neonatal sepsis of 88% and 98% versus 64% and 61% respectively. Cut off point for miRNA-16a for neonatal sepsis diagnosis was above or equal 3.16. Also, cut off point for miRNA-451 was above or equal of 1.26. In line with these observations, Wang and coworkers [1] investigated the level of multiple miRNAs, such as miRNA-15a, miRNA-16, miRNA-15b, and miRNA-451, in sepsis of neonates and demonstrated a significant difference in the miRNA-16a level between two groups; with significantly higher levels of MiRNA-16a in septic patients although there was no difference found in the MiRNA-451 level between two groups, the exact cause of such difference between our findings and Wang and coworkers [1] regarding miRNA- 451 is not clear. However, this difference can be clarified by the variability in the characteristics and demography of the included patients. The difference in sample size may be another cause. In adult population, Wang and his colleagues [18] investigated serum levels of miRNA-15a and miRNA-16a in sepsis patients and systemic inflammatory response syndrome (SIRS) without infection. Serum miRNA-15a ($p < 0.001$) and miRNA-16a ($p < 0.05$) were both

Table 4
Comparison between the two studied groups regarding miRNA- 451.

MiRNA- 451	Cases (n = 25)	Control (n = 25)	p
Min. – Max.	0.49–6.24	0.30–3.15	
Median (IQR)	1.43 (1.15–2.41)	1.25 (0.55–1.44)	0.034*

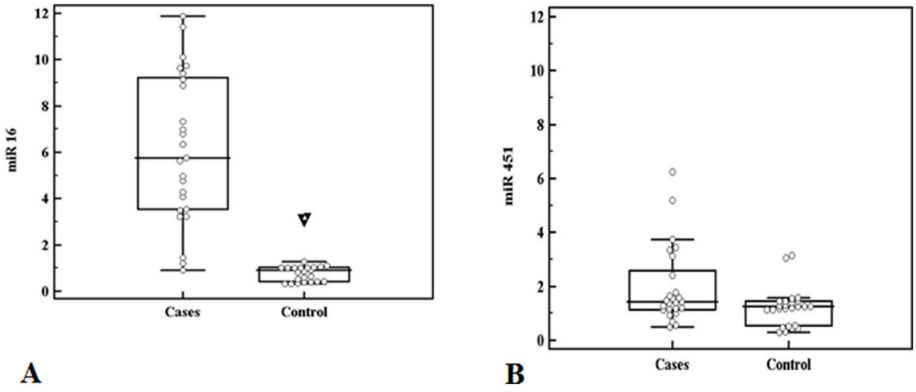

Fig. 4. (A) Comparison between the two studied groups according to miR-16a serum levels. (B) Comparison between the two studied groups according to miRNA-451 serum levels.

Table 5
Validity test for MiRNA- 16a and MiRNA- 451 to differentiate septic cases from controls.

	AUC	P	95% C·I	Cut off	Sensitivity	Specificity	PPV	NPV
MiRNA- 16a	0.968	<0.001*	0.924–1.012	>3.164	88.0	98.0	95.7	88.9
MiRNA- 451	0.674	0.034*	0.525–0.824	>1.262	64.0	60.0	61.5	62.5

AUC: area under curve, PPV: positive predictive value, NPV: negative predictive value.

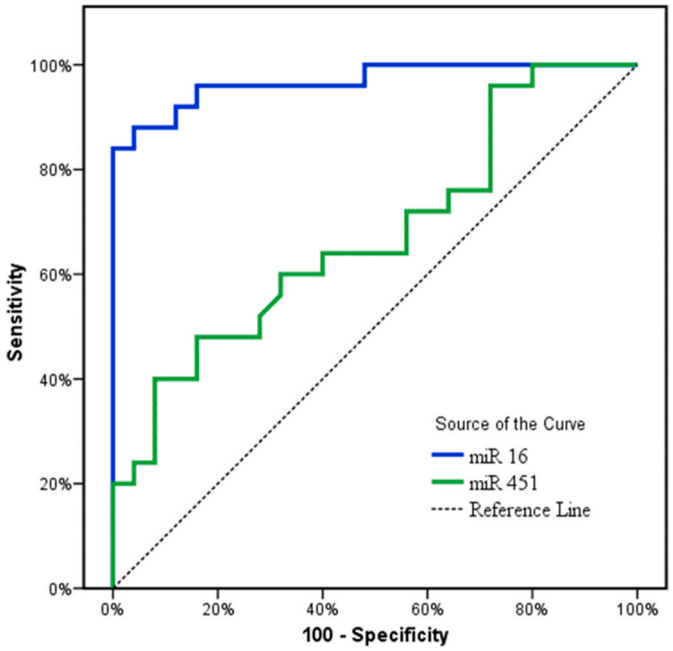

Fig. 5. ROC curve for different parameters for forecasting cases (vs control) in order to differentiate between septic patients and healthy controls.

Table 6
Correlation between miRNA-16 and miRNA-451 with different parameters in cases group (n = 25).

	miRNA 16		miRNA 451	
	rs	p	rs	p
Heart rate	0.321	0.117	-0.094	0.656
Respiratory rate	0.639	0.001*	0.472	0.017*
Temperature	0.009	0.966	0.024	0.910
Weight	-0.017	0.935	-0.032	0.879
Respiratory	0.052	0.806	-0.149	0.476
RD downes score	0.007	0.972	-0.198	0.343
WBC	0.623	0.001*	0.490	0.013*
HB	-0.138	0.510	0.107	0.612
PLT	0.053	0.800	-0.003	0.990
CRP	0.448	0.025*	0.468	0.018*

substantially higher in sepsis patients as opposed to normal controls. ROC curves showed that miRNA-16a had sensitivity of 68.3% and a particularity of 94.4%. As regards the prognostic performance of miRNA- 16a and miRNA-451 in neonatal sepsis, we discovered that both miRNAs correlated positively with proxies of severe presentation such as respiratory rate, leukocytic count, and CRP. Respiratory distress patients exhibited higher rate of miRNA-16a and miRNA- 451. These results were confirmed in univariate regression i. such results can highlight the significant prognostic feature of those biomarkers in sepsis. Colleagues and Wang [19] found that miRNA- 16a was a prognostic predictor for patients with sepsis. The AUC was 0.953 (95% CI: 0.923–0.983), The predictive value had a sensitivity of 88.5% and a precision of 90.4 with a cut-off point of 0.550.

5. Conclusion

Septic neonates had substantially higher levels of level of miRNA-16a and miRNA- 451 than healthy neonates with miRNA-16a was superior to miRNA-451 concerning neonatal sepsis diagnosis with greater sensitivity and specificity. So, it could be considered as a possible biomarker for neonatal sepsis diagnosis. Both miRNAs correlated positively with proxies of severe presentation such as respiratory rate, leucocytic count, and C - Rate of reactive protein (CRP). Neonates with respiratory discomfort exhibited higher level of miRNA −16a and miRNA- 451. These results can highlight significant prognostic function of those biomarkers in neonatal sepsis.

CRediT authorship contribution statement

Sally M. El-Hefnawy: Writing - review & editing. **Rasha G. Mostafa:** Writing - original draft. **Rania S. El zayat:** Data collection. **Esraa M. Elfeshawy:** Sample collection. **Hamed M. Abd El-bari:** Supervision. **Manal Abd El-Monem Ellaithy:** Methodology, Investigation.

Declaration of competing interest

There is no conflict of interest among authors.

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Appendix A. Supplementary data

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