The value of circulating miR-16/34 a as potential molecular markers for diagnosis and prognosis of neonatal sepsis

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Abstract: **Background**: Neonatal sepsis displayed the different symptoms in comparison with that of children or adults sepsis because of the infant's immature immune system. It's not clear whether these mi RNA biomarkers can be used as fingerprints to evaluate the prognosis of neonatal sepsis, and there have been few studies on the evaluation of mi RNA level in infant sepsis, thus it is necessary to confirm whether these mi RNAs can function as biomarkers in neonatal sepsis patients. The aim of this work was to investigate the possible value of circulating micro RNAs 16/ 34 a in the diagnosis and prognosis of neonatal sepsis. **Methods:** This study was a case control study, which done on 100 neonates (80 cases and 20 neonates with age and sex matched to the first group and apparently healthy, considered as a control group). Cases group are subdivided into 2 groups culture +ve group (40 cases) and culture –ve group (40 cases). This study was done at full terms attending Neonatal Intensive care Unit (NICU) in Benha University Hospitals. All subjects were subjected to history, clinical examination and laboratory investigation including estimation of miR-16 and 34 a. **Results:** There was a significant association between maternal risk factors as PROM and UTI and sepsis (P<0.001 for both). 82.5% of culture +ve group mentioned PROM compared with only 25% of culture –ve group. Also, 72.5% reported UTI compared with 12.5% of culture –ve group. Poor neonatal reflexes, lethargy and temperature instability occurred ta higher percentages among culture +ve group than culture –ve one. These differences were statistically significant (P<0.05 for all). Median value of mi RNA 16 was significantly higher among culture +ve group than both culture –ve and the controls (P<0.001). Regarding mi RNA 34a, it was significantly lower among culture +ve group than both culture –ve group than controls. This difference was highly significant (P<0.001). 15% of culture +ve neonates died compared with 0% of culture –ve ones. This difference was statistically significant (P<0.05). **Conclusion:** ROC curve analysis shows that the studied markers can significantly (P<0.001) diagnose sepsis at cutoff values mi RNA 16 ≥353.6 and mi RNA 34a ≤9.87. UAC was 0.802 and 0.857 respectively. Median value of mi RNA 16 was significantly higher among culture +ve group than both culture -ve and the controls. Regarding mi RNA 34a, it was significantly lower among culture +ve than both culture -ve and controls.

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Key words: Neonatal sepsis - potential molecular markers - miR-16/34- outcome.

1. Introduction:

Sepsis is a type of systematic inflammatory response syndrome (SIRS) caused by the invasion of pathogens or conditional pathogenic bacteria into the blood circulation. It can develop into severe sepsis, septic shock, and multiple organ failure ***(Zou et al., 2014).***

Neonatal sepsis is an important cause of mortality for neonates and remains a clinical challenge especially for asymptomatic infants. Observing these babies is crucial and any symptoms or abnormal laboratory result will prompt transfer to NICU (***Tarik Zahouani et al., 2017).***

It is estimated that four million neonatal deaths occur worldwide every year, and approximately one third of these are caused by infections. Sepsis and bacterial meningitis continue to be one of the main causes of neonatal mortality, especially among very low birth weight newborn infants **(Lona Reyes et al., 2015).**

Neonatal sepsis is a blood infection mainly caused by bacteria that occurs in an infant within 28 days old. Neonatal sepsis is categorized as early-onset or late-onset. Early-onset sepsis takes place in the first 3 days of life, while late-onset usually occurs at 4-28 days of life. The pathogens causing the neonatal sepsis majorly include Group B Streptococcus (GBS), Escherichia coli, Coagulase-negative Staphylococcus, Haemophilus influenza, and Listeria monocytogenes etc **(Klinger et al., 2009)**.

The incidence of neonatal sepsis in the developed countries is 1-8 per 1000 newborns, yet it is approximately three times in developing countries **(Thaver and Zaidi, 2009).**

The early diagnosis and timely management of sepsis are known to be crucial in the reduction of sepsis-induced mortality. This indicates that the early differentiation between sepsis and non-infectious SIRS has a significant impact on outcome **(Bracho-Riquelme et al., 2008)**.

Considering that these diagnosis conflicts might jeopardise sepsis management, there were intense need for the search of a rapid, sensitive, and specific gold standard for the diagnosis of sepsis, differentiating sepsis from non-infectious SIRS, and predicting its severity and outcome. The idea of a "Biomarker" for sepsis was enthusiastic for these issues with a resulting hundreds or even thousands of publications that studied numerous molecules that were supposed to be related to sepsis. **(Endo et al., 2012)**.

MicroRNAs (miRNAs) are endogenous small RNAs of ~22 nt lengths that regulate gene expression via binding target mRNAs for cleavage or translational suppression **(Bartel, 2004).**

It is known that the neonatal sepsis displayed the different symptoms in comparison with that of children or adults sepsis because of the infant's immature immune system. It's not clear whether these miRNA biomarkers can be used as fingerprints to evaluate the prognosis of neonatal sepsis, and there have been few studies on the evaluation of miRNA level in infant sepsis, thus it is necessary to confirm whether these miRNAs can function as biomarkers in neonatal sepsis patients. **(Wang et al., 2015)**.

The aim of this work was to investigate the possible value of circulating micro RNAs 16/ 34 a in the diagnosis and prognosis of neonatal sepsis.

2. Subjects and Methods:

This study was a case control study, which done on 100 neonates (80 cases and 20 neonates with age and sex matched to the first group and apparently healthy, considered as a control group). Cases are subdivided into culture +ve group (40 cases) and culture –ve (40 cases) group. This study done at fullterms attending Neonatal Intensive care Unit (NICU) in Benha University Hospitals, in the period from 2016 to October 2017.

Inclusion criteria:

Newborns were eligible for the study when they fulfilled the following criteria:

1. Fullterm infants
2. Both sexes
3. Birth body weight: appropriate for gestational age

Exclusion criteria

1. preterm infants
2. Infants with chromosomal anomalies
3. Infants with HIE
4. Incomplete clinical data or deviation from the study protocol presence or abscence of features sugestive of sepsis and the laboratory investigstions that confirmed or excluded sepsis diagnosis:

❖ **Group I** (Patient group) (80 cases): which was classified into 2 groups

✓ ***Group I-a*** (culture +ve group): which had the clinical features of sepsis and positive laboratory confirmation of septicemia

✓ ***Group I-b*** (culture–ve group) this group had clinical features suggesting sepsis but the laboratory investigation were negative

❖ **Group II** (Control group) (20 cases): had neither clinical features nor laboratory evidence of sepsis.

The following investigations were done to septic neonates

1. Complete blood count (CBC) with differential: white blood cell count (WBC), Imature-to-total-Neutrophil ratio (I/T), platelet count.
2. Serum C-reactive protein (CRP)
3. Blood culture
4. Urine culture if needed
5. Serum glucose level
6. Imaging studies: chest x Ray, abdominal and cranial sonography if needed
7. Estimation of miR-16 and 34 a and some target genes in blood

Blood samples for micro RNAs determination were collected before and after treatment:

One blood sample for blood group. Blood samples were collected in EDTA vacutainer tubes and the blood was frozen at – 80 c for quantification of miR-16 and 34 a and some target genes. This Relative Quantitation of microRNA-16 and 34a was done by two-step Real Time PCR using SYBR Green. Relative Quantitation (RQ) using comparative C_T describes the change in expression of the nucleic acid sequence (target gene) in a test sample relative to the same sequence in a calibrator sample ***(Livak and schmittgen, 2001)***.

Statistical analysis:

The collected data were tabulated and analyzed using SPSS version 16 soft ware (Spss Inc, Chicago, ILL Company). Categorical data were presented as number and percentages. Chi square test (X^2), or Fisher's exact test (FET) were used to analyze categorical variables. Quantitative data were tested for normality using Kolomogrov Smirnove test assuming normality at $P>0.05$. Quantitative data were expressed as mean ± standard deviation, median and range. Student "t" test was used to analyze normally distributed variables among 2 independent groups, or Man Whitney U test for nonparametric ones. Difference among 3 independent means was analyzed using ANOVA for parametric variables or Kruskal

Wallis test (KWT) for non parametric ones. Spearman's correlation coefficient (rho) was used to assess correlation between non parametric variables. ROC curve was used to detect cutoff values of miRNAS with optimum sensitivity and specificity in early diagnosis and prediction of diagnosis of sepsis. The accepted level of significance in this work was stated at 0.05 (P <0.05 was considered significant), P value >0.05 is non significant (NS), P<0.05 is significant (S), P≤0.001 is highly significant (HS).

3. Results:

Table (1) Socio-demographic characters of the studied groups

Variable		Culture +ve group (N=40)		Culture -ve group (N=40)		Controls (N=20)		X^2	*P*
		No.	%	No.	%	No.	%		
Sex	Male	25	62.5	21	52.5	14	70.0	1.87	0.39 (NS)
	Female	15	37.5	19	47.5	6	30.0		
		Mean ±SD	Range	Mean ±SD	Range	Mean ±SD	Range	ANOVA	*P*
Age (days)		7.9±2.9	4-14	9.1±2.4	7-15	7.3±3.5	2-14	2.96	0.056 (NS)
GA (w)		37.8±0.36	37-38	37.6±0.49	37-38	37.7±0.47	37-38	2.69	0.073 (NS)
Weight (kg)		3.28±0.38	2.8-4.05	3.20±0.24	2.9-3.8	3.09±0.12	2.95-3.3	2.63	0.077 (NS)

Table (2) Comparing the studied groups of patients regarding clinical findings of sepsis score

Variable		Culture+vegroup (N=40)		Culture -ve group (N=40)		X^2	P
		No.	%	No.	%		
Lethargy	No	15	37.5	25	62.5	5.0	0.025 (S)
	Yes	25	62.5	15	37.5		
Poor Neo reflexes	No	0	0.0	5	12.5	FET	0.055 (NS)
	Yes	40	100.0	35	87.5		
Temp. instability	No	19	47.5	30	75.0	6.37	0.012 (S)
	Yes	21	52.5	10	25.0		
GIT manifestations	No	26	65.0	30	75.0	0.95	0.33 (NS)
	Yes	14	35.0	10	25.0		
RD	No	14	35.0	25	62.5	6.05	0.014 (S)
	Yes	26	65.0	15	37.5		

Table (3) Comparing the studied groups regarding CBC

Variable	Culture positive group (N=40)			Culture negative group (N=40)			Z_{MWU}	P
	Mean	± SD	Range	Mean	± SD	Range		
HCT	41.8	6.23	32-50	42.6	9.51	25.5-55.3	0.92	0.36 (NS)
Hb	12.8	1.31	11.1-14.8	14.2	3.78	7.8-19.7	2.06	0.04 (S)
WBCs	20.7	16.12	4.15-57.5	11.2	4.66	4.9-20.3	3.3	0.001 (HS)
PLTs	260.0	139.9	80-498	195.1	33.00	139-236	0.56	0.58 (NS)
I/T ratio	0.35	0.05	0.3-0.4	0.3	0.07	0.2-0.4	2.88	0.004 (S)

Table (4) Comparing the studied groups regarding miRNA 16 and miRNA 34a

Variable	Culture +ve group (n=40)		Culture -ve group (n=40)		Controls (n=20)		KWT	P
	Median	Range	Median	Range	Median	Range		
miRNA 16	75804.7*†	0.44-12626924.9	107.7	0-2748094.1	0.115	85877.9	27.3	<0.001 (HS)
MiRNA 34a	0.071*	0-7.36	1251.3*	0-468936.5	34598.4	0.54-1299927.7	41.3	<0.001 (HS)

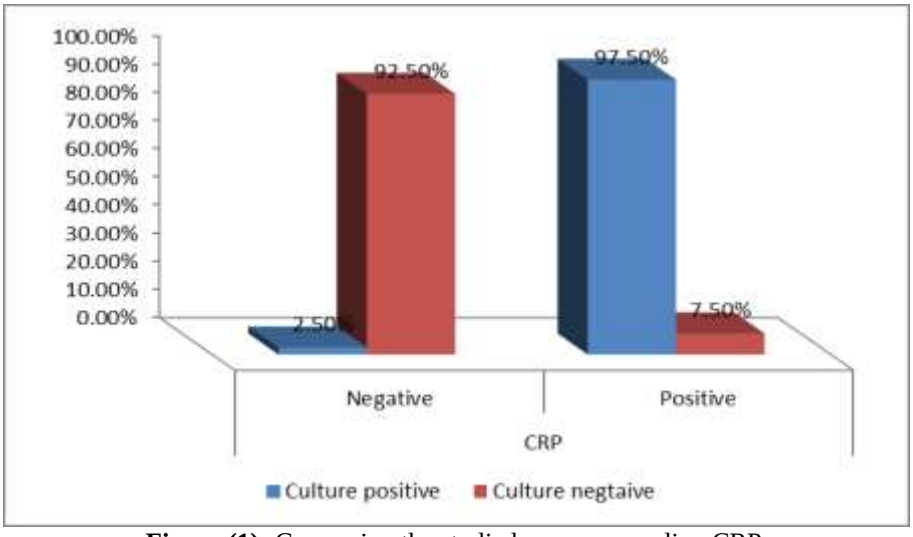

Figure (1): Comparing the studied groups regarding CRP

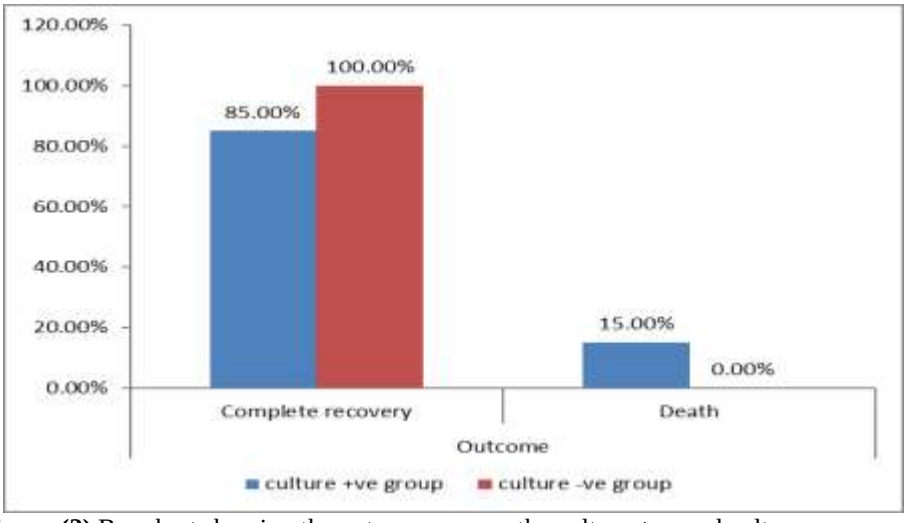

Figure (2) Bar chart showing the outcome among the culture +ve and culture –ve group.

Table (5) diagnostic performance of miRNA16 and miRNA 34a

Marker cutoff	Sens%	Spec%	PPV%	NPV%	Accuracy%	AUC	95%CI	P
miRNA 16 ≥353.6	82.5%	70%	64.7%	85.7%	75%	0.802	0.72-0.88	<0.001 (HS)
miRNA 34a ≤9.87	100%	78.3%	75.5%	100%	87%	0.857	0.78-0.94	<0.001 (HS)

Table (6) Comparison between the levels of the miRNA 16 and 34a according to outcome among culture +ve group

Variable	Survived (n=34)		Died (n=6)		Z_{MWU}	P
	Median	Range	Median	Range		
miRNA 16	75804.7	0.44-12626924.9	8480.9	8480.9-6140820.5	0.114	0.91 (NS)
MiRNA 34a	0.29	0-7.36	0.07	0.01-1.46	0.152	0.88 (NS)

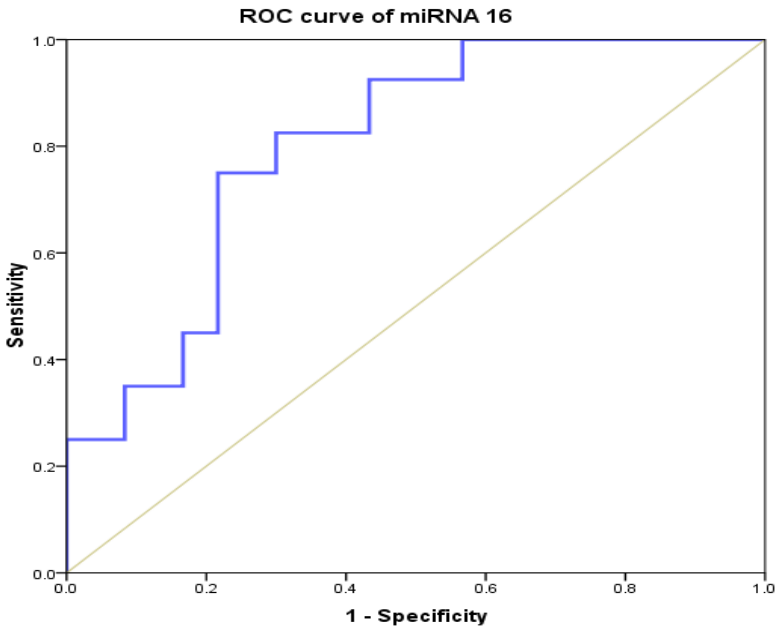

Figure (3) ROC curve OF miRNA 16

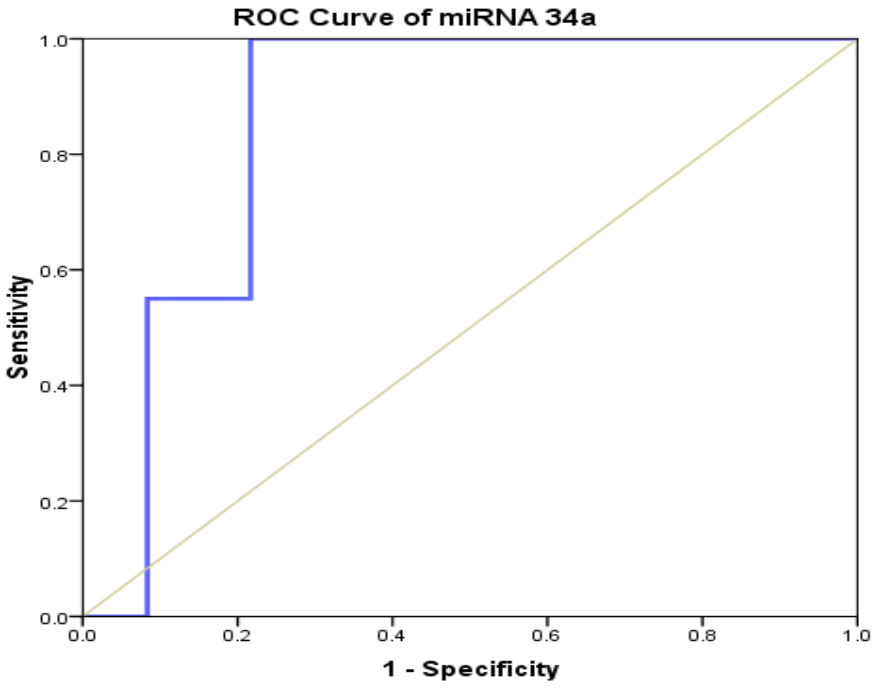

Figure (4) ROC curve of miRNA 34a

4-Discussion:

Our study was conducted on 80 septic fullterm newborns admitted in Neonatal Intensive care Unit (NICU) in Benha University Hospitals (group I) and 20 healthy fullterm newborns as controls (group II).

Septic newborns (group I) were subdivided into 2 subgroups: group I-a (culture +ve group) (40 cases) with clinical features of sepsis confirmed by positive laboratory findings and positive blood culture and group I-b (culture –ve group) (40 cases) with clinical features suggesting sepsis but blood culture was negative.

In the current study we found that there was no statistically significant difference between the studied

groups regarding socio-demographic characters (sex, age, GA and weight) as P value >0.05.

This is in accordance with the studies done by ***Wang et al., (2015)*** who investigated the level of miR 16 in both neonatal sepsis patients and control groups He found that there were no significant differences in the gender, age, gestational age and weight between the studied groups.

In our study, On clinical evaluation of the culture +ve group, poor neonatal reflexes, lethargy, and temperature instability were the commonest clinical presentations at early suspicion of sepsis (100%, 62.5%, and 52.5% respectively) this is in agreement with the study done by ***Fathy et al., (2009)*** who found poor Moro and suckling, and lethargy in 65%, 60%, and 55% respectively, that is slightly different than our results.

This is also similar to the results of the study done by ***Pradeep Verma et al., (2015)*** in which the most common clinical features were lethargy (71.6%), refusal to feed (67.5%) and fever (43.2%).

This disagrees with the study done by ***Shitaye et al., (2010)*** who found that the most prevalent clinical features of sepsis were hypothermia (84.8%), respiratory distress (72.8%), failure to feed (71.5%) and lethargy (30.1%).

In our study, we used complete blood count with special emphasis on Hematological scoring system of ***Rodwell et al., (2012)*** and CRP assay for diagnosis of neonatal sepsis and compared these indirect tests with the definitive tests for diagnosis of neonatal sepsis (blood culture).

In the present study there was statistically significant difference between culture +ve group, culture –ve groups regarding Immature to Total Neutrophil Ratio (I/T Ratio) which is higher in the culture +ve group with mean ±SD (0.35±0.05) compared to (0.3±0.07) in culture –ve group with P value < 0.004.

This agrees with the study done by ***Muhammad Rifky Ersadian et al., (2017)*** which was Based on the research results of Immature to Total Neutrophil Ratio (I/T Ratio) in normal neonates and neonates with neonatal sepsis at Ulin General Hospital Banjarmasin period April-June I/T Ratio in normal neonates has a mean value of 0.06, neonates with neonatal sepsis risk has a mean value of 0.15 and there was a significant difference between them.

Walliullah et al., (2009) found that the sensitivity and specificity of I/T ratio were 70% and 56%, respectively, in neonatal sepsis.

In our study, WBCs in the culture +ve group were significantly high with mean ± SD (20.7 ± 16.12) compared to (11.2±4.66) in culture –ve group with P value <0.001.

This agrees with the study done by ***Ahmed and Mahmoud, (2015)*** who found that, there was significant increase of WBCs in study culture +ve neonates when compared to culture –ve group (22.29±1.25 vs 9.10±1.29 respectively).

This is in disagreement with the study done by ***James et al., (2015)*** who did not identify statistically significant alterations in circulating WBCs between the studied groups (septic and control groups). This finding substantiates the documented limitations of WBC and WBC indices to identify infected infants.

Regarding Platelet count in our studied groups, there was not any significant difference between the culture +ve and culture –ve groups as the mean ± SD was (260.0 ±139.9) among the culture +ve group and (195.1±33.00) among the culture –ve group.

This is in accordance with the study done by ***Isabelle et al., (2017)*** who stated that thrombocytopenia is independently associated with neonatal sepsis.

This disagrees with the studies done by ***Medhat et al., (2017)*** in which platelet count showed a statistically significant difference between cases and control (232.7 vs 377.7) respectively.

In the present study, miR-16 was significantly higher among culture +ve group with median ± range (75804.7 ± 0.44-12626924.9) compared to median ± range of (107.7 ± 0-2748094.1) in culture –ve group and median ± range of (0.115 ± 85877.9) in controls and this was of high statistical significance with P value <0.001.

This is in accordance with the results of the study done by ***Wang et al., (2015)*** who found that, the transcription levels of miR-16 of neonatal sepsis patients were higher than control group. In detail, the mean relative miR-16 level increased 3 times in comparison with control. The results indicated that miR-16 might be the appropriate biomarker for diagnosing the Neonatal Sepsis.

This is also in accordance with the results of the study done by ***Huang et al., (2014)*** who identified many miRNAs that can serve as biomarkers for neonatal sepsis as: miRNA-15b, miRNA-16, miRNA-210, miRNA-324-3p, miRNA-484, miRNA-486-5p, miRNA-340, and miRNA-324-3p.

This is also in accordance with the results of the study done by ***Goodwin et al., (2015)*** who reported that notable several miRNA species, including miR-126, miR-21, miR-16, and miR-27a, increased more than 30-fold in neonatal sepsis.

This is also in accordance with the study done by ***Wang et al., (2012)*** who reported that miR-15a/16 play such a pivotal role in the inflammatory response, they can be evaluated as a potential biomarkers for early diagnosis of neonatal sepsis.

IN our study miRNA 34a was significantly lower among culture +ve with median ± range (0.071 ± 0-7.36) than both culture –ve group with median ± range (1251.3 ± 468936.5) and controls in which median ± range (34598.4 ± 0.54-1299927.7). This difference was of high statistical significance with P value <0.001.

Similarly a study done by ***Goodwin et al., (2015)*** found that, miRNA 34a, was significantly lower among neonatal sepsis group than controls.

In our study to define the cutoff value of miRNA for the diagnosis of neonatal sepsis and the associated specefity and sensitivity levels, a receiver operating characteristic (ROC) analysis was performed in the present study.

ROC analysis showed that the studied markers can significantly diagnose sepsis (P <0.001) at cutoff value for miRNA 16 ≥ 353.6 with sensitivity (82.5%), speceficity (70%) and AUC 0.802.

This is in accordance with results of the study done by ***Wang et al., (2015)*** who found that, the AUCs miR-16 was 0.8688 and found The AUC for miR-16 had the highest value, followed by that for miR-15a, and both displayed dramatic statistical differences in septic neonates compared to controls.

In the present study regarding miRNA 34a, ROC analysis shows that the studied marker miRNA 34a can significantly diagnose sepsis (P<0.001) at a cutoff value ≤ 9.87 with sensitivity 100%, specificity 78.3% and AUC 0.857.

This agrees with the study done by ***Goodwin et al., (2015)*** who found that, ROC curve analysis shows that the studied markers including miRNA 34a can significantly diagnose sepsis with AUC was 0.78 and P value <0.001.

The current study showed that there was no significant difference in the level of miRNA 16 and 34a between survivors and dead newborns in the culture +ve group.

Our results are against the results of the study done by ***Wang et al., (2012)*** who reported that miR-16 was detected as a prognostic biomarker and it also showed differential expression between sepsis survivor and nonsurvivor neonates.

5-Conclusion:

According to the forementioned studies, we can suggest that serum levels of miRNA 16 is elevated during neonatal infection and miRNA34a is decreased during neonatal infection and can be used as diagnostic markers for neonatal septicemia. We suggest that the potential physiopathological, diagnostic, prognostic and threpeutic roles of miRNA 16 and miRNA34a in neonatal sepsis should be further investigated.

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