ORIGINAL ARTICLE

Ascitic Fluid Micro RNA 155 as a Biomarker for Spontaneous Bacterial Peritonitis

[1]Sahar M. Fayed, [1]Yasser M. Ismail, [2]Hala M. Elfeky, [1]Manal Mosalem*

[1]Clinical & Chemical Pathology Department, Faculty of Medicine, Benha University

[2]Hepatology, Gastroenterology and Infectious Diseases Department, Faculty of Medicine, Benha University

Key words:
Spontaneous Bacterial Peritonitis, Micro RNA 155, Leukocyte esterase test, MPV, CRP and culture

Corresponding Author:
Mosalem Manal
Clinical Pathology Department, Benha University of Medicine, Egypt
Tel.: 01003670010
manalmosalem@yahoo.com

ABSTRACT

Background*: Spontaneous bacterial peritonitis is a life-threatening complication in cirrhotic ascitic patients.* ***Objectives:*** *We aimed to evaluate ascitic fluid microRNA155 level as diagnostic markers of (SBP) in association with Leukocyte esterase test, MPV, PDW, ascitic fluid CRP and culure.* ***Methodology:*** *A cross sectional study included 64 patients with ascites admitted to the Hepatology, Gastroenterology and Infectious diseases Department, Benha University Hospital, Egypt, divided into two groups: SBP group (32 pts.) and non SBP group (32 pts.).50 ml. of ascitic fluid was obtained for assessment of CRP, Leukocyte esterase test, bacterial culture and miR-155 and 12 ml blood for the routine laboratory test.* ***Results****: The AUC of miRNA 155 level was acceptable for discrimination between both groups (AUC=0.959), with cut off values 1.14pg/ml. Sensitivity 93.8%, specificity 93,7%, the cut-off value of ascitic fluid CRP was ≥12 ng/ml could predict SBP with sensitivity 81.3 % and specificity 81.2% , the Leucocyte esterase test cut-off value grade 1 and above could predict SBP with sensitivity 96.9 % , specificity of 84. %, accuracy of 90.6% ,with (AUC) of 0.906, MPV and PDW could predict SBP with cut of ≥10.5% and 14.3fl , sensitivity of 84.4% and 59.4%% , and specificity of 84.4% and 62.5%% ,accuracy of 84.4 % and 60.9 %, the combined usage of rapid bed side tests including leucocyte esterase strips, ascitic fluid CRP, MPV and PDW could predict SBP in almost all cases* ***Conclusion****: MiR-155 Leukocyte esterase test, MPV, ascitic fluid CRP and culure were good diagnostic markers of SBP.*

INTRODUCTION

Cirrhosis as end-stage of any chronic liver injury, is characterized by fibrosis and distortion of vascular architecture, leading to increased hepatic resistance which together with splanchnic hyper perfusion contributes to ascites formation.[1] Ascites is often complicated by spontaneous bacterial peritonitis (SBP), most often caused by bacterial translocation from the intestine, which might cause renal failure and is associated with high mortality. [2]

It is usually monomicrobial infection and majority of cases are caused by enteric gram-negative organisms, mostly Escherichia coli. [3]

The gold standard test to diagnose SBP is a polymorphonuclear neutrophil count of ≥ 250/µl in ascitic fluid using a manual counting chamber, regardless of the outcome of ascitic fluid culture [4].

In the past two decades, several studies have examined the bedside diagnosis of SBP such as the use of leukocyte esterase reagent strips [5]. The concentration of C reactive protein (CRP) in serum has been found to be helpful in the diagnosis and management of patients with a wide variety of inflammatory conditions.[6] Also platlets indecies including MPV & PDW were significantly elevated in cirrhotic patients with SBP [7].

Micro RNA's (miR) have been recognized as important modulators of gene expression and potential biomarkers. However, they have been rarely investigated in bio fluids apart from blood [8].

MiR-155 is involved in macrophage activation, which is next to lymphocytes the predominant cell population in ascites [9].

The aim of this study is primarily to evaluate ascitic fluid Micro RNA 155 as a biomarker for SBP and secondarily to assess the usefulness of some bed side tests for early diagnosis of SBP and to determine the antimicrobial susceptibility pattern for the isolated pathogens.

METHODOLOGY

This cross sectional study was included sixty four adult cirrhotic patients with and without SBP from December 2019 to September 2020 who attended Hepatology, Gastroenterology and Infectious Diseases Department, Benha University Hospitals. Patients were divided into 2 groups:

- **SBP group (Group1)**: It included 32 cirrhotic ascitic patients, their ascitic fluid PMNLs count was ≥250 cell/mm³.
- **Non SBP group (Group2):** It included 32 cirrhotic ascitic patients their ascitic fluid PMNLs count was < 250 cell/mm³. Patients <18 years, patients with liver cancer, secondary bacterial peritonitis, patients who received antibiotics 2 weeks before enrollment in the study, concomitant severe infections and those with cardiac ,renal and pancreatic ascites were excluded from the study.

All patients were subjected to the following: thorough history taking, full general and local examinations and abdominal ultrasonography. Severity of liver disease was assessed by Child Pugh score. Routine laboratory investigation was done including: Complete blood count (CBC), ESR, Prothrombin time and international normalized ratio (INR). Blood chemistry was done including serum glucose level, liver panel such as Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins, serum albumin, serum bilirubin, kidney panel such as serum creatinine, blood urea, serum sodium and potassium.

C-reactive protein (CRP) by Spinreact CRP latex agglutination slide tests for both serum and ascitic fluid

Blood culture: When ascitic fluid infection was suspected clinically, BACT/alert adult standard bottles was inoculated with 6-10 ml blood under complete aseptic condition for aerobic bacterial growth and incubated in BACT/alert automated system (bioMérieux, france). Subculture was done on blood and MacConkey's agar plates. [10]

Diagnostic paracentesis

Aspirated ascitic fluid was examined for: color, turbidity, total leucocyte count by manual methods using a hemocytometer counting chamber, ascitic fluid PMN count by leishman stained smear with light microscope [11] ,total protein, albumin and glucose

Leukocyte esterase reagent strips: **Using Combi-screen 10SL leucocyte esterase reagent strips**, **(Germany).** [12]

Interpretation of test based on the degree of colour change in the reagent strip area, Negative, Grade 1, 2 and 3

Ascitic fluid C - reactive protein [(6)]: Using CRP-latex slide agglutination test, Spinreact, SPAIN

Microbiological examination: Ten mL of the ascitic fluid was and inoculated at bed side into aerobic BACT /ALERT FA Plus (USA) blood culture bottles and was loaded in Bac-T/Alert 3D (bioMérieux, france). Identification of isolated pathogen was done by colonial morphology, Gram staining and different Biochemical tests. [13]

Antibiotic susceptibility testing [14]: It was carried out by the Kirby-Bauer agar disk diffusion according to the recommendations of CLSI, 2019.

Quantification of microRNA-155 in ascitic fluid samples: [15]

The ascitic fluid samples collected were subjected to RNA extraction using Direct-zol RNA MiniPreP (Zymo Research, California, USA) RNA extraction kits. Gel electrophoresis, followed by spectrophotometer analysis was carried out to ensure the purity of the resulting RNA. One microgram of total RNA was reverse transcribed to synthetize its complementary DNA (cDNA) using the The miScript II RT Kit (cat. 218160, 218161) Qiagen, germany at 37°C for 60 minutes followed by incubation at 95°C for 5 minutes in BIO RAD system, (T100 Thermal cycler) Singaphore. PCR contained 10 µl SYBR Green Master Mix (Qiagen, Georgia, USA) and 5 µl cDNA in addition to 0.5 µl of each primer (100 pmol/µl) and the volume was adjusted to 20 µl by adding nuclease-free sterile water. The thermal profile was as follows: 95°C for 15 min, followed by 40 cycles We measured each sample in duplicate. Melting curves were constructed using the ABI-Prism California, USA) in optical-grade 96-well plates. Sequence Detection Software version 1.6.3 (step one Applied Biosystems, Germany) was used to analyze the resulting data. Normalization of results was performed to the cycle threshold (Ct) of Syncel-miR- 39 (internal control). The levels of expression of miR-155 were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical method

Data were analyzed using SPSS software, version 22.0 (IBM, Armonk, NY, USA). Categorical data were presented as number and percentages, Chi square ($\chi 2$) and Fisher's exact tests. Quantitative data were tested for normality using Shapiro-Wilks test assuming normality at $P>0.05$. Linear association between variables was assessed by Spearman's correlation coefficients. ROC curves were constructed to assess the validity and predictivity of the studied markers in early diagnosis of SBP.

RESULTS

This study was carried out on 64 patients ,33 female and 31 male with age ranged between 43 – 80 years, the mean age was 55 ±10 years.

No sex predominance was noticed between groups while in concerning with associated comorbidities, there was high statistical significant difference between studied groups for diabetes mellitus and hypertension ($p<0001$) (table 1).

The most common presentation in SBP group was encephalopathy (34.4%) followed by abdominal pain (21.9%), fever (18.8%) and abdominal enlargement (15.6 %.)

According to Child Pugh classification**,** 40.6% of SBP cases were class B and 59.4% were class C. There was significant statistical difference between both groups regarding liver and spleen size and presence of

internal echo in ascitic fluid by abdominal ultrasonography (P=0.006, 0.017 and 0.024 respectively).

There was high significant statistical difference between the studied groups regarding blood TLC, PMN, PMV, ESR and CRP (P<0.001). There was significant statistical difference between both groups regarding PDW and serum Na (p=0.005).

Table 1: Socio-demographic characters of SBP and non SBP groups:

Variable		SBP group (n=32)		Non SBP group (n=32)		Test of sig.	P
		Mean±SD	Range	Mean±SD	Range		
Age (ys)		55.9±10.7	38-85	56.5±8.8	35-72	St"t"=0.24	0.81 (NS)
		No.	**%**	**No.**	**%**		
Sex	**Male**	20	62.5	13	40.6	X^2=3.06	0.08 (NS)
	Female	12	37.5	19	59.4		
Associated comorbidity	**Non**	6	18.8	6	18.8	FET=16.3	<0001 (HS)
	HTN	3	9.4	17	53.1		
	DM	16	50.0	7	21.9		
	Both	7	21.9	2	6.3		

As regard Ascitic fluid analysis for the studied groups: There was high significant statistical difference between both groups regarding physical appearance, PMNs ,total protein, CRP, TLC, glucose level, miRNA155, leuckocytic esterase strips (p<0.001) (table 2).

Table 2: Ascitic fluid analysis for the studied groups

Ascitic fluid		SBP group (n=32)			Non SBP group (n=32)			Z_{MWU}	*P value*
		Median	IQR	Range	Median	IQR	Range		
CRP (ng/ml)		24	12-48	6-96	5.0	4-6	3-12	5.95	<0.001 (HS)
TLC (cells /mm^3)		1800	817.5-3200	300-40000	132.5	61.3-217.5	20-480	6.78	<0.001 (HS)
PMN/m (cells / mm^3)		850	550-2425	280-20000	10	1.3-47.5	0-120	6.89	<0.001 (HS)
T protein (g/dL)		2.3	1.6-3.4	1-8	1.5	1-2	0.6-4.6	3.8	<0.001 (HS)
Albumin (g/dL)		0.65	0.5-0.9	0.2-2.0	0.75	0.6-0.9	0.1-2.2	0.84	0.4 (NS)
Glucose (mg/dl)		50.0	30-73.8	10-150	90	71.3-100	45-116	4.32	<0.001 (HS)
MiR-155 (pg/ml)		3.6	2.5-5.8	0.1-8.0	0.4	0.19-0.78	0.01-1.8	6.32	<0.001 (HS)
			no	**%**	**no**	**%**	χ^2	**P**	
Appearance	**Turbid**		25	78.1	5	15.6	25.1	<0.001 (HS)	
	Clear		7	21.9	27	84.4			
Leukocytes esterase strip	**-ve**		0	0.0	27	84.4	60.7	<0.001 (HS)	
	G1		11	34.37	5	15.6			
	G2		8	25	0	0.0			
	G3		13	40.62	0	0.0			

Diagnostic performance of ascitic fluid inflammatory markers and platelet indices using the Roc curve for diagnosis of SBP taking ascitic fluid PMN count as a reference method revealed that ascitic fluid miRNA 155 at cut off 1.14 pg/ml had Sensitivity 93.8%, specificity 93,7%, accuracy 93.8 %, positive predictive value 93.8% and negative predictive value 93.8%. (AUC=0.959) and confidence interval of (0.9-1) (table 3 & fig1).

Table 3: Diagnostic performance of ascitic fluid inflammatory markers and platelet indices for diagnosis of SBP:

Marker	Cut off	Sensitivity%	Specificity%	PPV%	NPV%	Accuracy%	AUC	95%CI
MiR- 155	≥1.14	93.8%	93.7%	93.8%	93.8%	93.8%	0.959	0.9-1.0
Ascitic fluid CRP	≥12	81.3%	81.2%	81.3%	81.3%	81.3%	0.927	0.87-0.99
MPV(fl)	≥10.5	84.4%	84.4%	84.4%	84.4%	84.4%	0.888	0.8-0.97
PDW%	≥14.3	59.4%	62.5%	61.3%	60.6%	60.9%	0.686	0.55-0.82
Leukocyte estrase strip	Positive G1,2,3	96.9%	84.4%	86.1%	96.4%	90.6%	0.906	0.82-0.99
Combined CRP,MPV, PDW and leukocyte esterase strip		100%	100%	100%	100%	100%	1	1

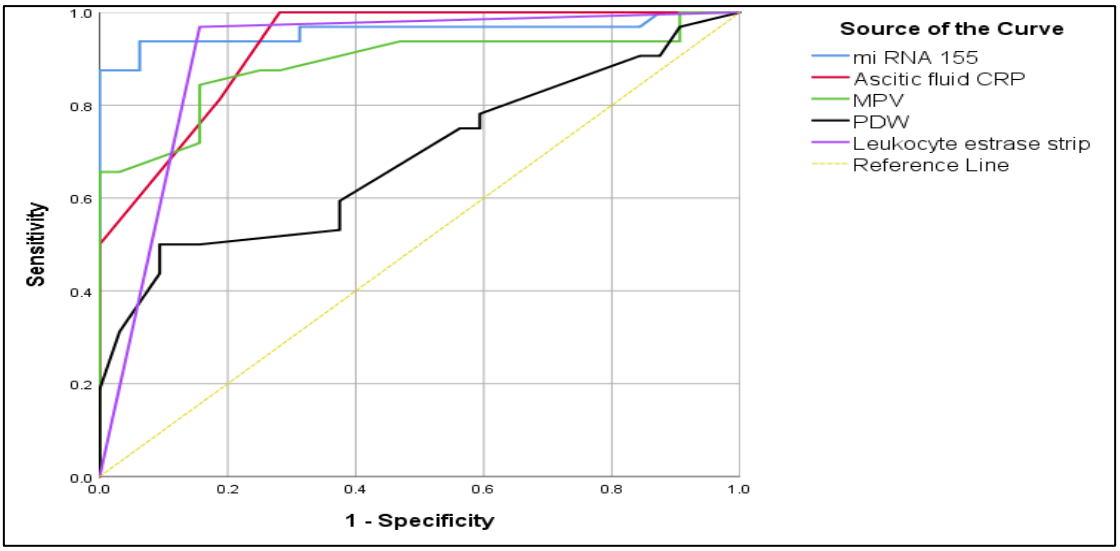

Fig. 1: ROC curve for the performance of some bed side tests with micro RNA 155.

The Roc curve showed that **ascitic fluid CRP** at cut off ≥12 ng/ml could predict SBP with sensitivity 81.3 % and specificity 81.2% , positive predictive value 81.3% ,negative predictive value 81.3%, accuracy 81.3% ,with area under the curve (AUC) of 0.927 and confidence interval of (0.87-0.99).

Regarding **Leucocyte esterase,** test ROC curve analysis demonstrated that the cut off value positive grade 1 and above had sensitivity 96.9 %, specificity of 84. %, positive predictive value 86.1 %, negative predictive value 96.4 %, accuracy of 90.6% ,with area under the curve (AUC) of 0.906 , and confidence interval of 0.82-0.99.

The curve also revealed that **MPV and PDW** cut off ≥10.5% and 14.3fl had sensitivity 84.4% and 59.4% , ,specificity 84.4% and 62.5%, positive predictive value 84.4% and 61.3 % , negative predictive value 84.4 % and 60.6% ,accuracy of 84.4 % and 60.9 %, with area under the curve (AUC) of 0.888 and 0.686 and confidence interval of 0.8-0.97 and 0.55-0.82 respectively. By curve analysis it was noticed that the usage of combined rapid tests including leucocyte esterase strips, ascitic fluid CRP, MPV and PDW could predict SBP in almost all cases (100%) in our study.

Ascitic fluid culture results using BACT/ALERT blood culture bottle were positive in 71.9% of SBP case. The most common isolated pathogen was E Coli (31.3%) followed by Klebsiella (12.5%) then S. aureus (no 3 -9.4%), Enterococci (no 3 -9.4%), CONS (no 2 -6%) and the least isolated organism was Pseudomonas (no1- 3.1%).There was 1 case of neutrocytic bactascitis that was Enterococci on microbiological culture while showing PMN count < 250 cell /mm^3.

In our work, blood culture in clinically suspected SBP cases was positive in 24.2% (8) in a total of 33 case and E coli was predominantly in (4) cases , Staph aureus in (3) cases and (1) was Enrterococci. They were also positive in ascitic fluid culture.

As regard antibiotic susceptibility pattern among isolated gram negative bacteria, Colistin was the most sensitive antibiotic 100% then Doxycline 93.3% then Cefepime and Amikacin 73.3% for each and for gram positive bacteria, linezolid and Vancomycin were most sensitive 100% then Doxycycline and gentamycin 77.7% for each.

Spearman correlation was done for ascitic fluid mi RNA155 level and different laboratory markers in SBP group, there was high significant positive correlation between ascitic fluid mi RNA155 level and MPV, ESR and CRP in blood (P =0.001), and significant positive correlation between ascitic fluid mi RNA155 level and blood TLC, PMN and PDW (P= 0.002, P= 0.004, P= 0.011) respectively (figure 2).

There was high significant positive correlation between ascitic fluid mi RNA 155 level and ascitic fluid Leukocyte esterase strips results (P<0.001), and significant positive correlation between ascitic fluid mi RNA 155 level and Ascitic fluid CRP, TLC and PMN (P=0.009, P=0.032, P=0.004) respectively.

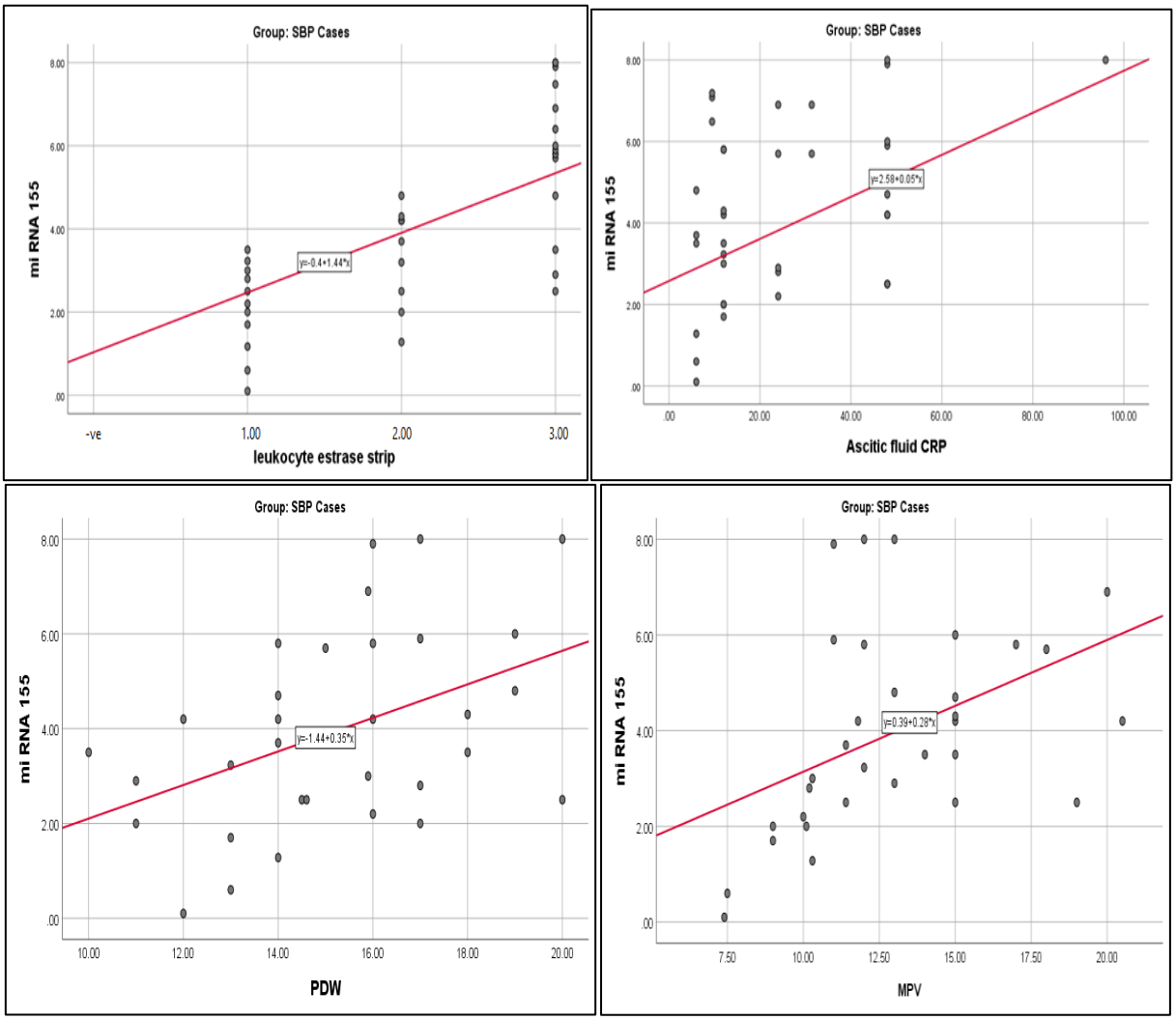

Fig. 2: Scatter graphs showing significant positive correlation between micro RNA 155 and inflammatory bed side tests (ascitic fluid CRP - MPV- PDW- leukocyte esterase strips).

DISCUSSION

Clinical manifestation of SBP is non-specific and variable, up to one third of patients might be asymptomatic (bactascites). [16]

MiR-155 is one of microRNAs (miRs) that are known to be small, noncoding RNAs of about 21–25 nucleotides. MiR-155 can exert their effect on liver inflammation and disease. [15]

In our study, there was no significant statistical difference between the studied groups regarding their age and sex that was in accordance with results of kassem et al [17.] Diabetes mellitus (DM) was higher in SBP group than non SBP group with high significant statistical difference (p<0001) that was agreed by Tergast et al [18] who found that DM was significantly associated with a higher incidence of recurrent SBP episodes.

In our study, Disturbed conscious level, abdominal pain, fever and abdominal enlargement were the main clinical presentation in SBP patients (34,4 %, 21% , 18.8% and 15.6% respectively). This result was in agreement with El Motasem et al. [19]

As regard routine laboratory findings of the current study, it showed no significant statistical difference between both groups except for TLC in blood, PMN, platelets indices (MPV and PDW) and serum Na. These results were in agreement with Khorshed et al. [7.]

In our study, CRP in blood with ESR levels were elevated in SBP group with high statistical significant difference (p<0.001) among both groups that agreed with Awad et al. [20,] this explained by activation of cytokine synthesis in response to circulating bacterial endotoxins as reported by El sadek et al., [21].

There were high significant statistical differences regarding AF total leucocytic count, PMNLs and ascitic fluid total protein between both groups with (p <0.001) Selim et al [22,] agreed with our results.

In our study, leucocyte esterase reagent strips were positive in (91 %) of SBP group with sensitivity of 96.9 % and specificity of 84.4 %.that agreed by Asmaa et al.[12] In contrary Nousbaum et al.[23] who elicited much lower sensitivity. It may be due to different types of the used strips.

CRP levels may be elevated in the ascitic fluid due to increased capillary permeability and as a result of the local infalamatory event in SBP group. Moreover, the clearance of CRP in the ascitic fluid was unknown Zalam et al., [6].

Ascitic fluid CRP level was high in SBP group with statistical significant difference among studied groups (p<0.001) that was agreed by Zalam et al. [6] In contrast Runyon [24)]reported no difference. This may be explained by the matter that CRP is produced in the liver and only enters a body cavity by leakage of serum protein.

In our study, we found that MPV and PDW could predict SBP at a cut of ≥10.5 fl and ≥14.3% with sensitivity of 84.4% and 59.4%% , specificity of 84.4% and 62.5%% and positive predictive value 84.4 % and 61.3 % , negative predictive value of 84.4% and 60.6 % , accuracy of 84.4 %and 60.9 % respectively that was agreed by korshed et al. [7]

Our work revealed that the specificity of MPV for detection of SBP was superior to PDW. This is in accordance with Suvak et al. [25]

In our study, miR- 155 was high in SBP group with high significant statistical difference (p>0.001) that was agreed with Nabiel et al.[15] The (ROC) curve analysis showed that the cut-off value of ascitic fluid miR-155 level was ≥ 1.14pg/ml that could predict SBP with sensitivity 93.8%, specificity 93.7%, positive predictive value 93.7%, negative predictive value 93.8% and accuracy 93.8%.

Lutz et al.[8] reported some agreement with our results. As they found that miR- 155 has sensitivity 90% while a low specificity of 37%, this discrepancy may be due to different sample size and age groups.

We demonstrated that concerning SBP group, there was high significant positive correlation between ascitic fluid miR- 155 level and most of inflammatory markers in blood including MPV, ESR, CRP in blood (P =0.001), blood TLC, PMN and PDW (P=0.002 ,P=0.004, P=0.011) respectively, that agreed with lutz et al. [8]

In our study, Ascitic fluid cultre using BACT/ALERT blood bottle was positive in 23 case (71.9%) of SBP cases and negative in 9 cases (28.1%) of cases. That result was agreed by Oladimeji et al [26] while, Hafez et al· [3] found only 40.3% of patients had culture-positive ascitic fluid. This may be due to different culture methods .Our study found that there was 1 case of bactascitis.,Hafez et al. [3)]found that 6 patients (9.7% of SBP patients) were bacterascites.

In our study, the most common isolated bacteria were E Coli (10 -31.3%) then Klebsiella (4- 12.5%) , S. aureus (no 3 -9.4%), Enterococci (no 3 -9.4%), CONS (no 2-6%) and Pseudomonas (no1-3.1%). As regard antibiotic susceptibility paterrn among isolated gram negative bacteria, Colistin was the most sensitive antibiotic 100% followed by Doxycline 93.3% followed by Cefepime and Amikacin 73.3%. While among gram positive bacteria, it was as follow, Linezolid and Vancomycin were most sensitive 100% followed by Doxycycline and Gentamycin 77.7% that was agreed with Ding et al. [27]

In our study, Blood culture in clinically suspected SBP cases was positive in 24.2% (8) in a total of 33 cases. E coli was predominant in 4 cases, Staph aureus in 3 cases and 1 case was Enterococci. They were positive in ascitic fluid culture. It was matched by Sanglodkar et al. [28.]

CONCLUSION

Ascitic fluid micro RNA 155 level is a good marker for SBP diagnosis. Combined use of leucocyte esterase strips test, ascitic fluid CRP and (PDW and MPV) is a rapid sensitive bedside tool for diagnosis of SBP.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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