

ORIGINAL ARTICLE

Evaluation of Ascitic Fluid Calprotectin as an Accurate Marker for Rapid Diagnosis of Spontaneous Bacterial Peritonitis in Patients with Chronic Liver Disease

¹Ahmed O. Shafik, ¹Ahmed M. Elgazzar, ²Ahmed S. Elgazar, ¹Marwa A. Elaraby*,
¹Mysa S. Mostafa

¹Department of Medical Microbiology and Immunology, Faculty of Medicine, Benha University, Egypt

²Department of Hepatology and Gastroenterology, Faculty of Medicine, Benha University, Egypt.

ABSTRACT

Key words:
Spontaneous bacterial peritonitis, Calprotectin, ELISA

***Corresponding Author:**
Marwa Ashraf Elaraby,
Department of Medical Microbiology and Immunology, Faculty of Medicine, Benha University, Egypt.
Tel: 01270148859
drmarwaelaraby@gmail.com

Background: Spontaneous bacterial peritonitis is the development of an infection of the ascitic fluid in the peritoneum, with no identifiable source of the infection in patients with liver failure. ascitic fluid neutrophil count is more than 250/mm³. calprotectin in Ascitic fluid may help in detection of neutrophil count < 250 cells/mm³, it has an effective role in detection of SBP and this will be a rapid treatment and good prognosis. **Objectives:** Evaluation of the calprotectin in ascitic fluid as rapid marker for diagnosis of SBP. **Methodology:** our study was done on 40 patients in Hepatology and Gastroenterology Department of Benha University divided into 2 groups 20 patients were SBP group and other 20 patients were non SBP group. 5ml of ascitic fluid was collected from patient in sterile blood culture bottles under complete aseptic condition then cultured on automated blood culture system (Bact/ALERT 3D) .Serum Calprotectin was measured in ascitic fluid by using commercially available quantitative sandwich enzyme-linked immune sorbent assays. **Results:** SBP was in significant association with higher TLC in AF and higher frequency of positive culture results when compared to non SBP group ($p < 0.001$ for each). Among all studied cases, median calprotectin level was 0.602 ng/dL. SBP was significantly associated with higher level of calprotectin when compared to non SBP group .Calprotectin showed positive significant correlation with TLC in ascitic fluid. **Conclusion:** calprotectin in ascitic fluid is an excellent rapid marker for diagnosis of spontaneous bacterial peritonitis in patients with chronic Liver disease.

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is the occurrence of peritonitis regardless the absence of source of infection that occurs mostly in cases of portal hypertension, due to liver cirrhosis e.g cholecystitis or pancreatitis¹.

Infection of ascitic fluid can be classified into five subtypes according to polymorphonuclear cell count and ascitic fluid culture results :

Classic culture positive SBP, culture negative SBP also called culture negative neutrocytic ascitic (CNNA), monobacterial non neurocytic bacterascites ,secondary peritonitis , polymicrobial buster-associates².

The most frequent subtype of ascitic fluid infection is culture-negative SBP, which occurs in approximately 30% to 50% of patients because the ascitic fluid culture is not positive in all SBP patients with ascitic fluid neutrophil count $\geq 250/\text{mm}^3$ ³ and also called 'culture-negative SBP' because bacteria are present in a lower concentrations and could not diagnosed by ordinary

microbiological culture methods⁴. So, the growth of bacteria in the ascitic culture does not detect presence of SBP, since bacteria are detected only in about 40% of SBP patients⁵.

It has been proven that delayed diagnosis of SBP is associated with bad prognosis. Thus, an accurate biomarker for the early identification of SBP would be of great diagnostic value. Ascitic fluid calprotectin may help in detection of neutrophil count > 250 cells/mm³, it has an great role in detection of SBP and this will be a rapid method beside test in quick treatment of SBP⁶.

Calprotectin is an abundant, calcium- and zinc-binding protein found mainly in neutrophils⁷ and its apperance in body fluids is proportional to the influx of neutrophil. it represents 60% of the soluble proteins in the cytosol. Lower concentrations are found in monocytes and active macrophages⁸.

Calprotectin is accurate, stable marker that is not changed by medication, dietary supplements, or enzymatic degradation. Calprotectin has antimicrobial properties and it can stop the proliferation of both

normal and malignant cells, probably by sequestration of zinc, which is a vital element for many enzymes⁹.

Calprotectin normally exists in human body fluids (e.g., plasma, synovial fluid, saliva, and urine) and faeces, and its level significantly increases in various infectious and inflammatory diseases e.g inflammatory bowel diseases. Then neutrophils are activated, leading to liberation of cellular proteins, including calprotectin. Calprotectin transports across the epithelial barrier and enters the lumen of the gut. So faecal calprotectin is an vital marker for diagnosis of inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis¹⁰.

METHODOLOGY

This study was done in the Microbiology and Immunology Department, Faculty of Medicine, Benha University at period from May 2020 to August 2021

The study was done on 40 patients admitted to the Hepatology and Gastroenterology Medicine Department, Benha University Hospital with ascites on top of liver cirrhosis diagnosed both clinically and ultrasonographically. The patients were two groups:(1) Group A: included 20 cases with SBP diagnosed by the clinical picture and ascitic fluid PMNL of 250/mm³ or more.

(2) Group B: included 20 patients without SBP diagnosed by the clinical picture and ascitic fluid polymorph nuclear cells less than 250/mm³.

Exclusion criteria

- Patients with secondary peritonitis and extra-hepatic malignancy were excluded.
- Patients who had received antibiotic within 3 weeks prior to admission.

All patients were subjected to the following:

Complete history taking, Full clinical examination, Laboratory investigations:

Routine tests :S. bilirubin (direct, indirect), SGOT and SGPT, S. Albumin, urea, creatinine, Complete blood count (CBC), Polymorph nuclear leukocytes in ascitic fluid (PMN), Viral markers including: HBsAg, HBcAb IgM and IgG and HCV Ab by third generation ELISA.

Specific tests:

- Sampling: Paracentesis (5 ml of ascitic fluid will be collected from patient in sterile blood culture bottle under complete aseptic condition).
- Bactec: Culture on automated blood culture system (Bact/ALERT 3D). The bottles were kept under

both anaerobic and aerobic conditions at 37C for about 5 days, according to BioMérieux company instructions. The culture bottle was labelled with patient information. The plastic flip-top was removed from culture bottle. Prior to inoculation, the culture bottle top was disinfected with an alcohol swab or equivalent and allowed to air dry. The inoculated culture bottles were put into the Bact/ALERT System as soon as possible after collection as recommended by Zhuhai Meihua Medical Technology Ltd. .If there is an unavoidable delay, inoculated bottles may be maintained at room temperature up to 24 hours before loading into the instrument. The inoculated culture bottles were incubated five to seven days or until designated positive.

- ELISA: Ascitic fluid calprotectin was measured by using of commercially available quantitative sandwich enzyme-linked immune sorbent assays. Samples were centrifuged during 15 min (3,500 rotations/min) and the supernatant was collected into clean tubes without any chemical or biological addition in the collection device, and stored refrigerated at 2–8°C for up to 7 days. For longer storage, samples were kept frozen at <20°C for a maximum of 3 months. This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). CAL was put in the wells pre-coated with CAL monoclonal antibody. After incubation a biotin-conjugated anti-human CAL antibody was added and binds to human CAL. After incubation unbound biotin-conjugated anti-human CAL antibody was washed away during a washing step. Streptavidin-HRP was added and binds to the biotin-conjugated anti-human CAL antibody. After incubation unbound Streptavidin-HRP was washed away during a washing step. Substrate solution was then added and color developed in proportion to the amount of human CAL. The reaction was terminated by addition of acidic stop solution and absorbance was measured at 450 nm¹¹

RESULTS

The most frequent symptoms of clinical presentation was abdominal pain (67.5%), followed by disturbed conscious level (40%), melena (32.5%), hematemesis (22.5%) and fever (20%), as seen in figure (1).

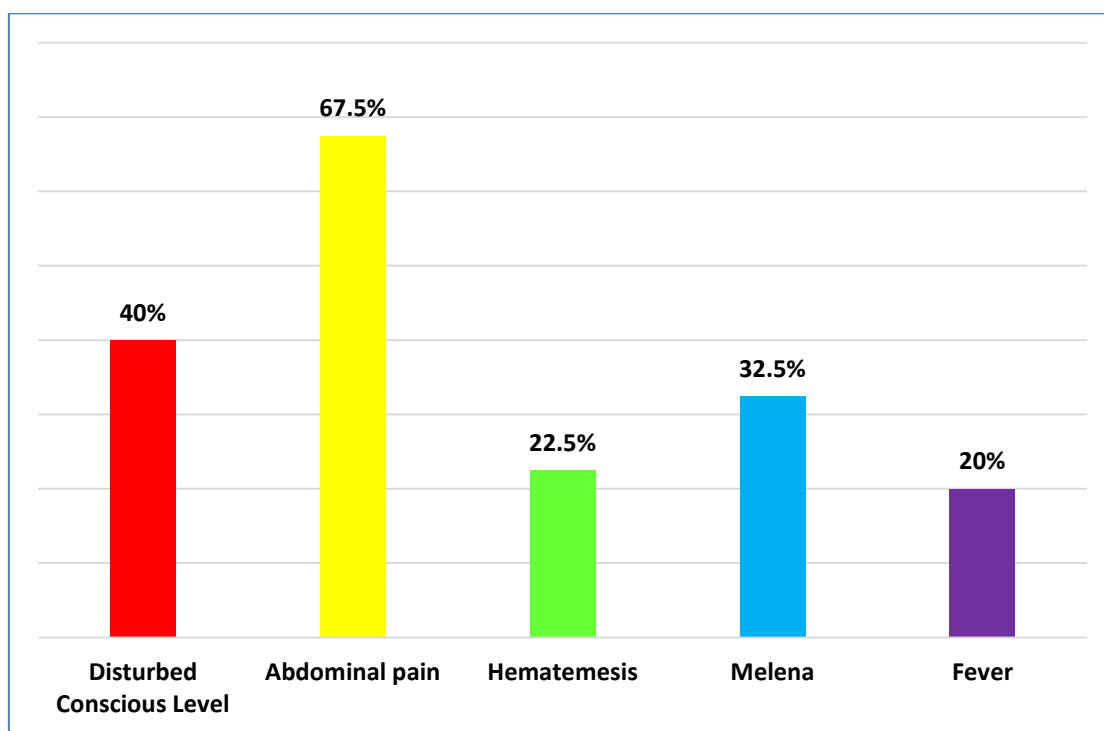


Fig. 1. Clinical data among studied cases.

No significant differences were found regarding blood laboratory parameters among studied groups, as shown in table (1).

Table 1. Comparison of blood laboratory parameters among studied groups.

		no SBP N=20		SBP N=20		P values
TLC (X10 ⁹ /L)	median (range)	9.5	(4.2-14.7)	10.75	(4.7-22.4)	
HB (g/dl)	median (range)	9.2	(6.5-11)	9.15	(6.3-11.8)	0.989
platelets (X10 ⁹ /L)	median (range)	168.5	(71-230)	175	(71-487)	0.675
Direct Bilirubin (mg/dl)	median (range)	0.95	(0.18-1.96)	1.15	(0.18-2.98)	0.239
Total Bilirubin (mg/dl)	median (range)	2.55	(0.7-3.9)	2.9	(0.7-5.7)	0.255
AST (U/L)	median (range)	23.5	(15-1103)	35.5	(12-332)	0.070
ALT (U/L)	median (range)	17.5	(10-857)	27.5	(11-115)	0.126
urea (mg/dl)	median (range)	102.5	(36.5-182)	107	(50-219)	0.685
creatinine (mg/dl)	median (range)	1.265	(0.6-2.1)	1.3	(0.5-2.98)	0.924
Albumin (mg/dl)	Mean±SD	2.2	±0.3	2.1	±0.3	0.292

SD, standard deviation; Mann Whitney test was used to compare data except albumin which was compared using t test.

SBP had significant association with higher TLC in AF and higher frequency of positive culture results when compared to no SBP group (p<0.001 for each), as shown in figure (2).

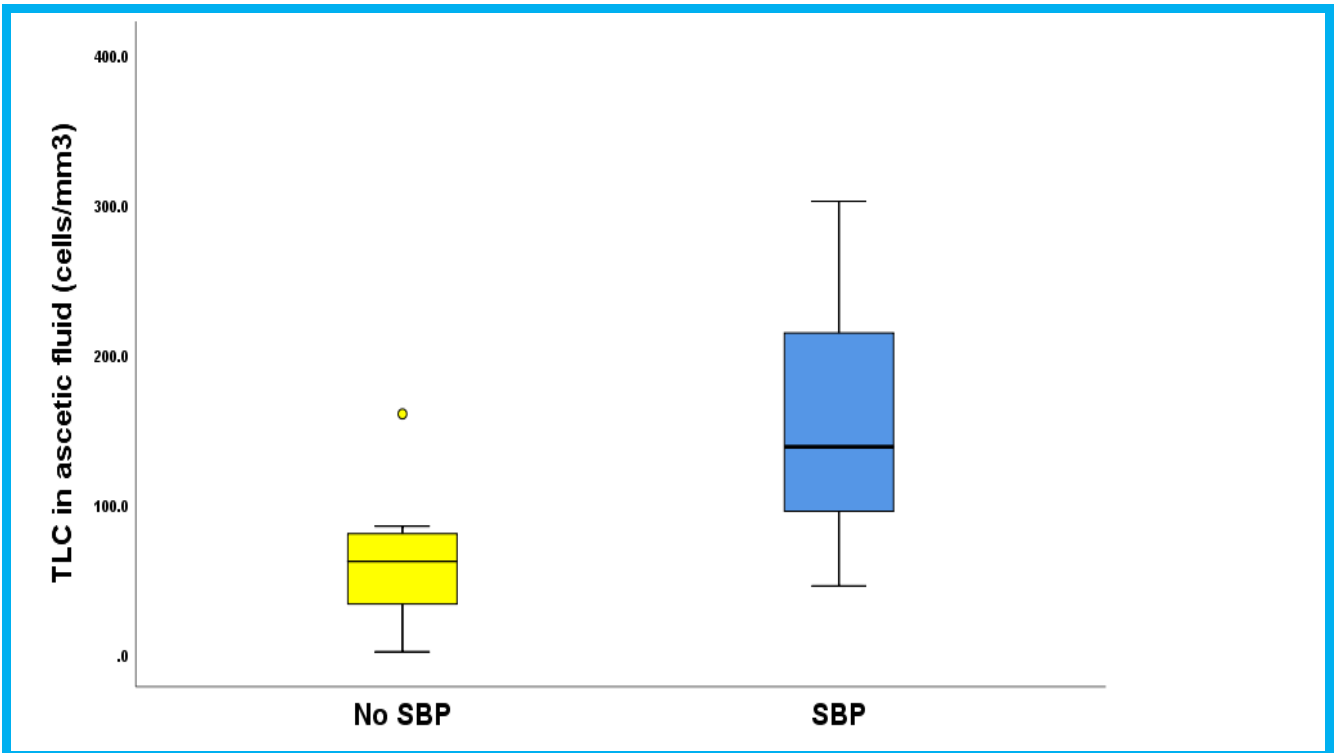


Fig. 2. TLC in AF among studied groups.

Among all studied cases, median calprotectin level was 0.602 ng/dL, ranged from 0.022 to 1.812 ng/dL. SBP was significantly associated with higher calprotectin when compared to no SBP group ($p < 0.001$ for each), as shown in figure(3).

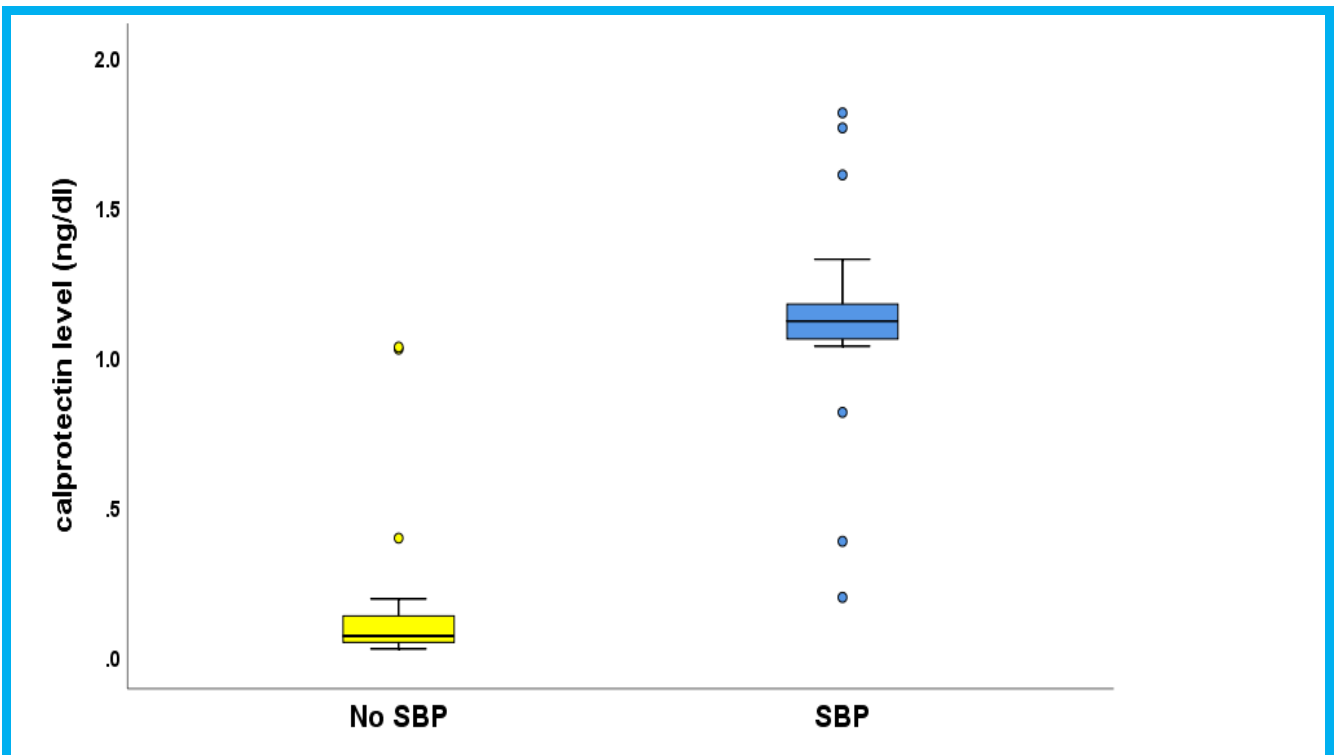


Fig. 3. Calprotectin level among studied groups.

ROC curve of blood, AF TLC and calprotectin was conducted for discrimination between patients with non infected ascites and those with SBP group.

High accuracy AUC was found for calprotectin (AUC=0.980), AF TLC had moderate accuracy AUC (AUC=0.895), while blood TLC had low accuracy AUC (AUC=0.643), as shown in figure (4).

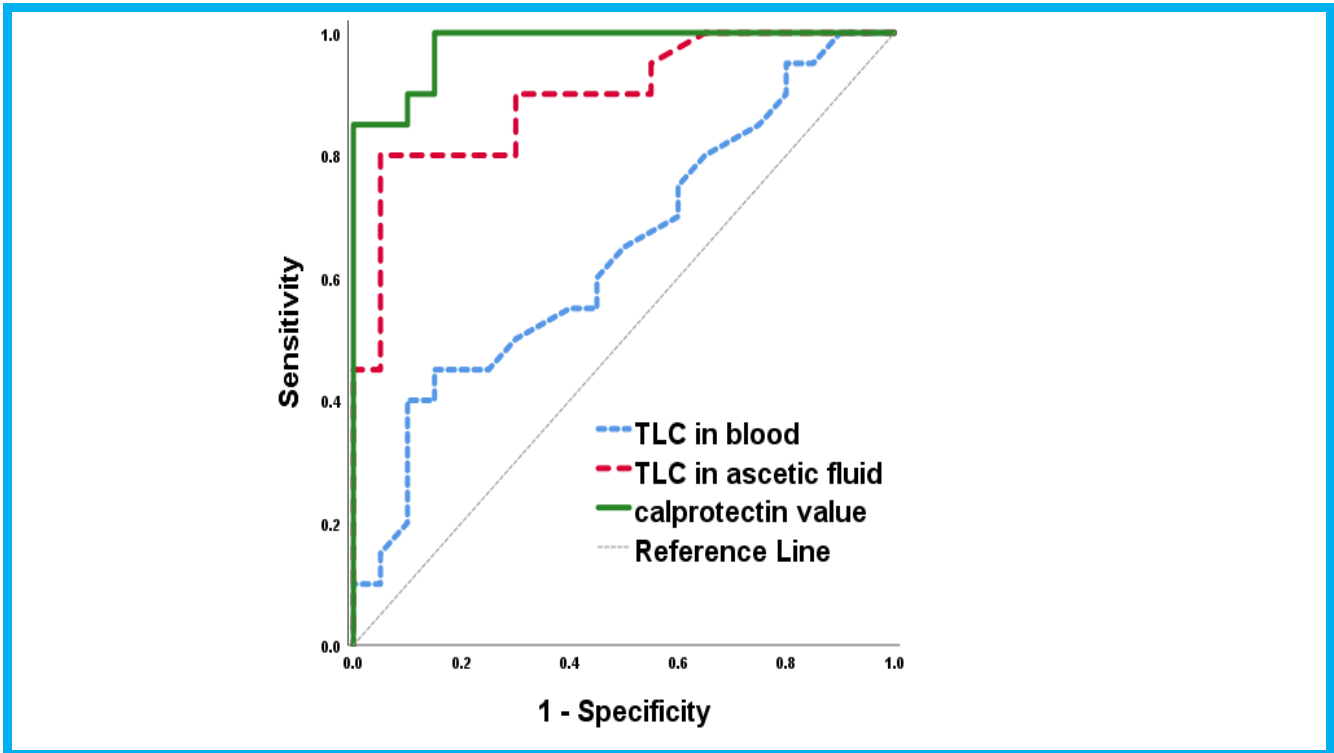


Fig. 4. ROC curve of blood, AF TLC and calprotectin for discrimination between patients with non infected ascites and those with SBP.

Regarding blood TLC, at best cut off value of $10.3 \times 10^9/L$, sensitivity was 55%, specificity was 60%, PPV was 57.9%, NPV was 57.1% and accuracy was 57.5%.

Regarding AF TLC, at best cut off value of $87.5/\mu L$, sensitivity was 80%, specificity was 95%, PPV was 94.1%, NPV was 82.6% and accuracy was 87.5%.

Regarding calprotectin, at best cut off value of 1.03, sensitivity was 85%, specificity was 100%, PPV was 100%, NPV was 87% and accuracy was 92.5%.

It is obvious that performance characteristics increased gradually from blood TLC, to AF TLC, and the highest performance characteristics were owned to calprotectin.

DISCUSSION

Liver cirrhosis is the one of serious complications of chronic liver disease when patients suffer from considerable mortality and morbidity, both of which are correlated positively with disease severity¹².

SBP is a serious complication in cirrhotic patients with ascites. Late or misdiagnosed SBP can lead to increased mortality; SBP affects 10-30% of cirrhotic patients hospitalized with ascites, and mortality in this group approaches 30%¹³.

The diagnosis of SBP still depends on PMNL count in AF of 250 cells/mm³ or higher in the absence of obvious source of intra-abdominal infection with or without a positive culture¹⁴.

Many markers in AF as calprotectin have been investigated for diagnosis of SBP¹⁵.

The aim of work is to evaluate calprotectin as immunological marker in ascitic fluid for rapid detection of SBP in cirrhotic patient.

The mean age of studied cases was 62.8 years. They were 21 males (52.5%) and 19 females (47.5%). This result was in agreement with the study done by Angeli et al.¹⁶ who reported that, SBP was frequent in males and was not affected by the age, while Puri et al.¹⁷ had reported that age does not affect the incidence of SBP.

Regarding clinical data, our study showed that the most frequency symptom of clinical presentations was

abdominal pain (67.5%), followed by disturbed conscious level (40%), melena (32.5%), hematemesis (22.5%) and fever (20%).

SBP was in significant association with higher frequency of abdominal pain when compared to non SBP group ($p < 0.001$). Otherwise, no significant differences were found regarding clinical data among studied groups ($p > 0.05$ for each). This result was in agreement with results that were reported by Llovet et al.¹⁸ who reported that, abdominal pain was detected in 52% of SBP cases. But, Wallersted et al.¹⁹ stated that, abdominal pain was found in 70% of SBP cases.

Parameters of laboratory investigations, In our study, no significant differences were found regarding blood laboratory parameters among studied groups. This result was in disagreement with that reported by Rodriguez et al.²⁰ who detected leucocytosis in their SBP cases with significant difference when compared to non SBP cases.

Regarding liver function tests, no statistically significant differences were recorded in all parameters. Casafont et al.²¹ found no significant difference in liver biochemistry. These results were in agreement with those reported by Runyon et al.²² who stated that, there is no relationship between hypoalbuminemia and SBP, but rather to the underlying liver disease.

In our study, there was no significance between renal function tests and SBP and this was in disagreement with Angeloni et al.²³ who concluded that the high mortality of SBP is due to renal impairment, which is common in SBP patients either due to prerenal or hepatorenal causes.

Regarding Correlation between TLC AF and AF culture by bact/alert, In our study, SBP was in significant association with higher TLC in AF and higher frequency of positive culture results in compression to no SBP group ($p < 0.001$ for each).

In our study, SBP was significantly associated with higher calprotectin when compared to no SBP group ($p < 0.001$ for each). Among all studied cases, median calprotectin level was 0.602 ng/dL, ranged from 0.022 to 1.812 ng/dL. This was in agreement with those reported by Burri et al.²⁴, Fernandes et al.²⁵, Elbanna et al.²⁶, Ali et al.²⁷, and Abdel-Razzik et al.²⁸.

Regarding AF TLC, at best cut off value of 87.5/ μ L, sensitivity was 80%, specificity was 95%, PPV was 94.1%, NPV was 82.6% and accuracy was 87.5%.

Regarding calprotectin, at best cut off value of 1.03, sensitivity was 85%, specificity was 100%, PPV was 100%, NPV was 87% and accuracy was 92.5%.

Fernandes et al.²⁵ concluded that the patients with evidence of SBP had significantly higher levels of ascitic fluid calprotectin than patients without SBP with a sensitivity, a specificity, a PPV, and an NPV of 87.8, 97.9, 97.3, and 90.2%, respectively.

Also, Abdel-Razzik et al.²⁸ had tested AF calprotectin for rapid diagnosis of SBP in 79 patients

with ascites and liver cirrhosis because of different etiologies unlike our patients who only had HCV, and stated that the ascitic calprotectin level was significantly higher; using a cutoff value of 470 ng/ml, the sensitivity, specificity, PPV, and NPV were 95.4, 85.2, 71, and 93%, respectively.

Burri et al.²⁴ reported that the best cutoff value of ascitic fluid calprotectin measured by the ELISA method for the diagnosis of SBP was 630 ng/ml with a sensitivity, a specificity, a PPV, an NPV, and an accuracy of 94.8, 89.2, 60, 99, and 90%, respectively.

The results of this study were in disagreement with that reported by Rito Norbe et al.²⁹ who found that, the diagnostic accuracy of Calprotectin are limited in ascitic fluid infection.

CONCLUSION

The ascitic fluid Calprotectin is an important marker for rapid diagnosis of spontaneous bacterial peritonitis in chronic Liver disease patients

Automated blood culture system is an excellent method in term of speed and sensitivity for detection of spontaneous bacterial peritonitis.

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.

REFERENCES

1. Sagar KV, Reddy PR and Chandrasekhar S. A clinical study of spontaneous bacterial peritonitis in cirrhosis of liver with ascites in tertiary care hospital. *J Evid Based Med Health*. 2016; 3(1), 36-41.
2. Enomoto H, Inoue S, Matsuhisa A, et al. Development of a new in situ hybridization method for the detection of global bacterial DNA to provide early evidence of a bacterial infection in spontaneous bacterial peritonitis. *J Hepatol*. 2012; 56:85-94.
3. Bibi S, Ahmed W and Arif. Clinical, Laboratory and Bacterial Profile of spontaneous bacterial peritonitis in cirrhotic liver disease patients. *Journal of the College of Physicians and Surgeons Pakistan*. 2015; 25(2): 95-99.

4. Ayling RM, Kok K. Fecal calprotectin. In: Makowski GS, editor. *Advances in clinical chemistry*. Amsterdam, The Netherlands: Elsevier. 2018; p.161-90.
5. Jalan, R, Fernandez, J, Wiest, R, Schnabl, B, Moreau, R, Angeli, P, Stadlbauer, V, Gustot, T, Bernardi M and Canton, R. Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference. *Journal of hepatology*. 2014; 60, 1310-1324.
6. Lutz P, Pfarr K, Nischalke HD, et al. The ratio of calprotectin to total protein as a diagnostic and prognostic marker for spontaneous bacterial peritonitis in patients with liver cirrhosis and ascites. *Clin Chem Lab Med*. 2015; 53:2031–2039.
7. Burri E and Beglinger C. The use of fecal calprotectin as a biomarker in gastrointestinal disease. *Expert Rev Gastroenterol Hepatol*. 2014; 8:197–210.
8. Burri E, Schulte F, Muser J, Meier R and Beglinger C. Measurement of calprotectin in ascitic fluid to identify elevated polymorphonuclear cell count. *World J Gastroenterol*. 2013; 19:2028–2036.
9. Ingvar T. Bjarnason, Roy A. Sherwood. *Clinical Biochemistry: Metabolic and Clinical Aspects (Third Edition)*. 2014; 214-230.
10. Pous-Serrano S, Frasson M, Cerrillo E, Beltran B, Iborra M, Hervas D et al. Correlation between fecal calprotectin and inflammation in the surgical specimen of Crohn's disease. *J Surg Res*. 2017; 213: 290–7.
11. Bioassay Technology Laboratory ELISA kit.
12. Moorthy S, Chalasani V, Ramakrishnan SR, Vasanthan K. Efficiency assessment of leucocyte esterase reagent strips in rapid bedside diagnosis of spontaneous bacterial peritonitis – a comparison study with the gold standard absolute neutrophil counts in ascitic fluid. *Indian J Appl Res*. 2015; 5:465–467.
13. Fernandes SR, Santos P, Fatela N, Baldaia C, Tato marinho R, Proenca H, et al. Ascitic calprotectin is a novel and accurate marker for spontaneous bacterial peritonitis. *J Clin Lab Anal*. 2016; 30:1139–1145.
14. Lutz P, Pfarr K, Nischalke HD, et al. The ratio of calprotectin to total protein as a diagnostic and prognostic marker for spontaneous bacterial peritonitis in patients with liver cirrhosis and ascites. *Clin Chem Lab Med*. 2015; 53:2031–2039.
15. Shizuma T. Diagnostic laboratory markers for spontaneous bacterial peritonitis. *Ann Clin Lab Res*. 2016; 4:131.
16. Angeli K.L, Moore KP, Moreau R. Management of ascites, spontaneous bacterial peritonitis, and hepatorenal Syndrome. *World J Gastroenterol*. 2000; (12), 232_312.
17. Puri AS, Puri J, Ghoshal UC, Sharma, B.C, Saraswat, VA, Ayyagari A et al. Frequency, microbial spectrum and outcome of spontaneous bacterial peritonitis. *Indian J Gastroenterol*. 1996; 15(3): 86-89.
18. Llovet J, Moitinho E, Sala M, Bataller R, Rodríguez Iglesias P, Castells A et al. Prevalence and Prognostic value of hepato cellular carcinoma in Cirrhotic patients presenting with spontaneous Bacterial peritonitis. *J. Hepatology*. 2000; 33: 423-29.
19. Wallersted S, Olsson R, Simern M, Broomé U, Wahlin S, Löf L et al. Abdominal tenderness in Ascites patients indicates spontaneous bacterial Peritonitis. *Eur. J. Internal medicine*. 2007; 18: 44-47.
20. Rodriguez-Ramos C, Galan F, Diaz F, Costaner A. Expression of pro inflammatory cytokines and their Inhibitors during the course of spontaneous bacterial Peritonitis. 2001; 46, 8, 1668 – 76.
21. Casafont F, Coworkers C, Fernande MD. Overview on cirrhotic patients with spontaneous Bacterial peritonitis .*Dig. Dis. Sci*. 1991; 44(10):1985-89.
22. Runyon BA, Akriviadis EA, Sattler FR, Cohen J. Ascitic fluid and serum cefotaxime and desacetyl Cefotaxime levels in patients treated for bacterial Peritonitis. *Dig. Dis. Sci*. 1991; 12 : 1782 – 1786.
23. Angeloni S, Leboffe C, Parente A ,et al. Efficacy of current guidelines for the treatment of spontaneous bacterial peritonitis in clinical practice. 2008; 14:2757-2762.
24. Burri E, Schulte F, Muser J, Meier R and Beglinger C. Measurement of calprotectin in ascitic fluid to identify elevated polymorphonuclear cell count. *World J Gastroenterol*. 2013;19:2028–2036.
25. Fernandes SR, Santos P, Fatela N, Baldaia C, Tato marinho R, Proenca H, Et al. Ascitic calprotectin is a novel and accurate marker for spontaneous Bacterial peritonitis. *J Clin Lab Anal*. 2016; 30:1139–1145.
26. Elbanna A, Allam N, Hossam N, Ibrahim A, Wagdy M. Plasma and ascitic Fluid level of calprotectin in chronic liver disease malignant and non-malignant. *Alexandria Bull*. 2008; 24:647–653.
27. Ali AG, Ahmed NS, Hasan SM. Calprotectin measurement in ascitic fluid: a new test for the rapid diagnosis of spontaneous bacterial peritonitis. *Med J Cairo Univ*. 2013; 81:53–56
28. Abdel-Razik A, Mousa N, Elhammady D. Ascitic fluid calprotectin and Serum procalcitonin as

- accurate diagnostic markers for spontaneous Bacterial peritonitis. *Gut Liver*. 2016; 10:624–631.
29. Rito Norbe S, Pina Cabral J.E, Sofia C, Correia Leitao M. Value of reagent strips and calprotectin role in the rapid diagnosis of spontaneous bacterial peritonitis. *Hepato-gastroenterology*. 2008; 55(84): 1020-1023.