Ascitic Fluid Lactoferrin as a Diagnostic Marker for Spontaneous Bacterial Peritonitis

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Background and study aim: Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in patients with cirrhosis and ascites. The diagnosis of SBP is established when the ascitic fluid polymorphonuclear leukocyte (PMN) counts is ≥250 cells/ mm3 with or without a positive ascitic fluid culture but this test lack sensitivity. The objective of this study was to evaluate the possible role of lactoferrin in diagnosis of SBP.

Patients and Methods: The study included seventy patients with liver cirrhosis and ascites admitted to hospital. Patients were classified into SBP group and control group by ascetic fluid PMN count. Aspirated ascitic fluid samples were examined for bacteriological culture, biochemical assay, and cytological count. Ascitic fluid lactoferrin was measured by ELISA technique.

Results: Ascitic fluid lactoferrin was significantly increased in SBP patients compared to control group. There was a statistically significant positive correlation between lactoferrin levels and PMN counts in SBP patients ($p<0.001$). ROC curve was used to determine a cutoff value for lactoferrin in diagnosis of SBP. At lactoferrin level ≥270 ng/ml, the sensitivity was 96%, specificity was 95%, positive predictive value was 97.96%, negative predictive value was 90.5%, and accuracy was 95.7% in diagnosis of SBP.

Conclusion: Measurement of ascitic fluid lactoferrin could serve as a rapid and reliable screening tool for diagnosis of SPB.

INTRODUCTION

Cirrhotic ascites forms as the result of a particular sequence of events. Development of portal hypertension is the first abnormality to occur. [1]. Hypoalbuminemia and reduced plasma oncotic pressure favor the extravasation of fluid from the plasma to the peritoneal fluid and thus ascites is infrequent in patients with cirrhosis unless both portal hypertension and hypoalbuminemia are present [2].

It is an important cause of morbidity and mortality in patients with cirrhosis and ascites, which identified in 10%-30% of hospitalized ascitic patients [3] and mortality can approach 30% [4]. Bacteria participating in SBP come from the digestive tract. Extra-intestinal bacteria are much less frequent. The development of SBP thus depends on the antibacterial capacity of ascitic fluid which is positively correlated to the content of the total protein in ascitic fluid and the immune-competence of the patient. The organism reacts to the infection by activating neutrophilic granulocytes which migrate into the peritoneal cavity and trigger a complex cytokine cascade. So, there are four key elements of SBP pathogenesis: small intestinal bacterial overgrowth, increased intestinal permeability, bacterial translocation and immune-suppression. These key elements are not separate, but interlinked [5].

The diagnosis of SBP is established when the ascitic fluid polymorphonuclear leukocyte (PMN) count is ≥250 cells/ mm3 with or without a positive ascitic fluid culture [6]. Lysis of the PMNs during transport to the laboratory may lead to false negative results. Manual measurement of the ascitic fluid PMN count is operator–dependant makes quality control difficult and can delay the diagnosis [7]. The use of urinary reagent strips has been proposed for rapid diagnosis of SBP. The urinary strips identify leukocytes by detecting their esterase activity via a colorimetric reaction. However, a large multicenter study suggested a lack of sensitivity of strip tests for the diagnosis of SBP and indicated an absence of diagnostic efficacy for this test [8].

Lactoferrin is an iron binding protein that is found mainly in external secretions such as breast milk and in PMNs and is released on degranulation [9]. Previous studies showed that lactoferrin in stool provide a reliable marker of inflammatory diarrhea [10]. The measurement of ascitic fluid lactoferrin could provide a reliable biomarker for the presence of PMNs and detection of SBP in patients with cirrhosis [3]. Our objective was to evaluate the possible role of lactoferrin in diagnosis of SBP.

PATIENTS AND METHODS

Seventy cirrhotic patients with ascites who were admitted to Tropical Department, Zagazig University Hospitals were included in this study. The diagnosis of liver cirrhosis and ascites was based on clinical, biochemical and ultrasonographic findings. Patients were classified into 20 patients with ascitic fluid PMN count <250 cells/mm³ (control group) and 50 patients with ascitic fluid PMN count ≥250 cells/mm³ (SBP group). SBP group was further subdivided into culture negative SBP& culture positive SBP. Bacteriological culture using aerobic and anaerobic standard blood culture bottles containing brain-heart infusion broth, which were inoculated with 10 mL of ascitic fluid and incubated for 48 hours at 37°C. None of the patients had received antibiotics for ten days prior to hospital admission. Patients with evidences of secondary bacterial peritonitis, tuberculous peritonitis or malignant ascites were excluded. Further exclusion criteria were ascites due to other causes e.g. cardiac, renal diseases or Budd-Chiari syndrome. All participants provided written informed consent after receiving oral and written information concerning the study.

All studied patients were subjected to medical history taking, clinical examination, routine laboratory investigations and abdomino-pelvic ultrasonographic examination. Aspirated ascitic fluid samples were immediately examined for bacteriological culture and identification of microorganisms, cytological count (manual), and biochemical assays (Cobas 501, Roch Diagnostics). Ascitic fluid albumin was measured using Albumin Latex Biosystem kit. Ascitic fluid lactoferrin was determined using Assay Max Human Lactoferrin ELISA Kit (Endomedx). This assay employs a quantitative sandwich enzyme immunoassay technique. The minimum detectable level is 0.1 ng/ml. Intra-assay and inter-assay coefficient of variation are 4.1% and 7.1% respectively.

Statistical Analysis

Statistical analysis was performed with SPSS software (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for the Microsoft Windows. Kolmogorov-Smirov test was used to verify the normality of distribution of continuous variables. Data are presented as mean ± standard deviation (SD) for continuous variables, frequency and percentage for categorical ones. Differences between the studied two groups were evaluated by independent sample t test and $\chi 2$ respectively. Fisher exact- test was used for comparisons between qualitative categories when there is an observed cell < 5. Bivariate correlations were performed using the Pearson correlation to determine correlation of lactoferrin to the different studied variables. The test results were considered significant when P value < 0.05. Receiver operator characteristic (ROC) analysis, area under curve (AUC) and 95% confidence interval (CI) were used to determine the optimum cutoff value of lactoferrin in diagnosis of SBP. Diagnostic performance was represented using the terms sensitivity, specificity, positive predictive value, negative predictive value, and accuracy.

RESULTS

Demographic and clinical manifestations of the studied groups are presented in table 1. Most of patients had Hepatitis C virus (HCV) infection which represent 75% in control group and 84% in SBP group, followed by bilharzias (15% in

control group and 10% in SBP group), then mixed HCV and bilharzias (10% in control group and 6% in SBP group), with no statistically significant difference between the two groups (p>0.0.05). Table 2 represents cytological and biochemical characteristics of the studied groups. Ascitic fluid lactoferrin was significantly increased in SBP patients compared to control group (p=0.001).

Most of SBP patients showed negative culture results (70%), while 30% showed positive results of E.coli, Klebsiella, Staphylococcus and Pseudomonas (18%, 8%, 2% and 2% respectively). Culture results were negative in control group (100%). There was no statistically significant difference in the studied laboratory parameters including lactoferrin between culture positive and culture negative SBP patients (p>0.05).

Correlations between ascitic fluid lactoferrin and the other studied laboratory parameters in SBP group are represented in table 3, PMN counts, glucose A/S ratio, and LDH A/S showed significant correlations. Ascitic fluid lactoferrin levels were significantly correlated with PMN counts in SBP patients (r=0.56, p<0.001) (figure 1). Analysis of ROC-AUC revealed AUC of 0.995 (95% CI: 0.985-1.005) (figure 2). At cutoff value ≥ 270 ng/ml, lactoferrin can detect 48 out of 50 SBP cases. Nineteen out of 20 control subjects had lactoferrin levels <270 ng/ml. Ascitic fluid lactoferrin had 96% sensitivity, 95% specificity, 97.96% positive predictive value, 90.5% negative predictive value, and 95.7% accuracy in diagnosis of SBP.

Table (1): Demographic and clinical manifestations of the studied groups.

Parameter	Control Group N=20	SBP Group N=50	P
Age (years)	55.4±7.2	52.8±7.1	0.17
Sex (M/F)	12/8(60/40)	25/25(50/50)	0.31
No symptoms	13(65)	8(16)	< 0.001 *
Fever	2(10)	24(48)	0.003 *
Abdominal Pain	2(10)	23(46)	0.005 *
Encephalopathy	2(10)	21(42)	0.008 *
Jaundice	2(10)	10(20)	0.265
Splenomegaly	3(15)	30(60)	0.001 *

N: number of subjects. Data are represented as numbers (frequencies) or mean ± SD *Significant.

Table (2): Cytological and laboratory characteristics of the studied groups.

Parameter	Control Group N=20	SBP Group N=50	P
Leucocytes (/mm^3)			
Total	159.5±72.4	4150.7±1202.3	<0.001*
PMN	33.2±13.9	3451.2±1148.2	<0.001*
Ascitic protein (g/dL)	2.41±0.66	1.64±0.54	<0.001*
Albumin (mg/dL)			
Ascitic	10.86±2.6	3.65±0.36	0.002*
Serum	2.18±0.35	2.51±0.45	0.047*
SAAG	2.3±0.48	2.14±0.36	0.128
Glucose (mg/dL)			
Ascitic	154.6±51.3	101.5±32.8	0.005*
Serum	152.3±42.6	114.2±31.9	0.062
A/S ratio	1.1±0.36	0.9±0.29	0.048*
LDH (IU/L)			
Ascitic	106.1±37.3	424.9±154.8	0.002*
Serum	481.6±136.8	562.6±126.2	0.148
Ratio	0.23±0.14	0.67±0.21	<0.001*
Ascitic lactoferrin (ng/mL)	162.5±65.3	3400.5±1177.1	0.001

N: number of subjects. Data are represented as mean ± SD. *Significant. A/S: ascitic fluid/serum

Table (3): Correlations between ascitic lactoferrin and other studied laboratory parameters in SBP patients.

Parameter	r	P
PMNs	0.56	<0.001*
Ascitic total protein	0.25	0.086
SAAG	-0.40	0.004*
Glucose A/S ratio	-0.61	<0.001*
LDH A/S ratio	0.79	<0.001*

*Significant.

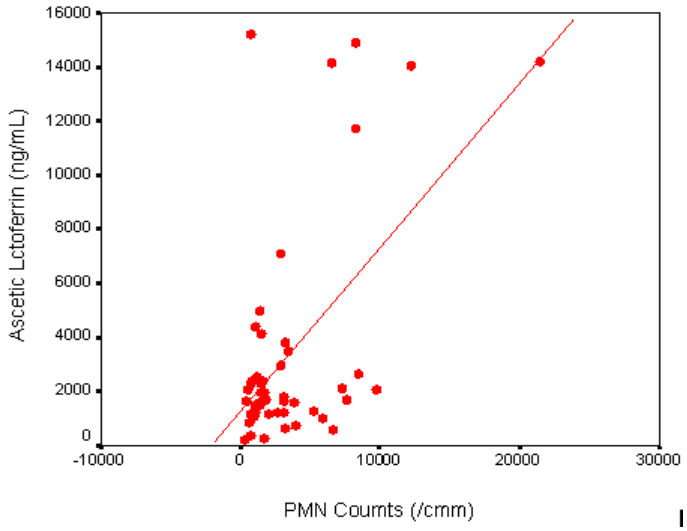

Figure (1): Correlation between ascitic fluid lactoferrin and PMN count in SBP group.

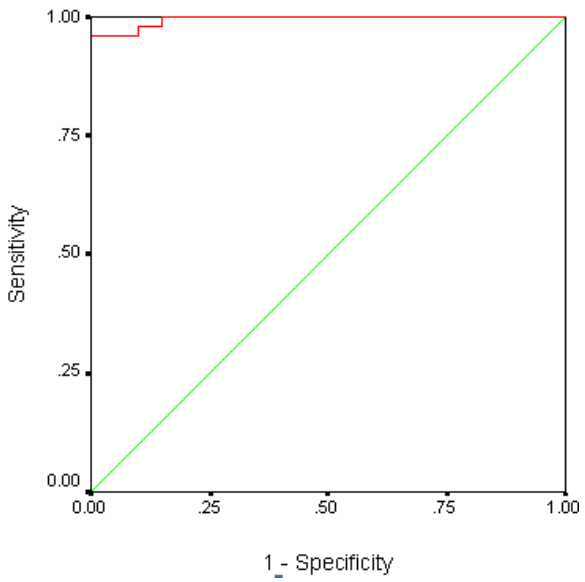

Figure (2): ROC curve analysis of ascitic lactoferrin for diagnosis of SBP. AUC of 0.995 (95% CI: 0.985-1.005)**.**

DISCUSSION

The clinical picture of SBP is non-specific and variable, mainly depending on the stage at which SBP is diagnosed [11]. The absence of clinical manifestations in some patients with SBP makes the dependence on a reliable marker is an important target taking into consideration that SBP is one of the most frequent and important complications found in cirrhotic patients with ascites [12]. In hospital, mortality rate can reach 30% in spite of infection control measures [13]. In the present study, the clinical features among the patients of SBP were not specific and asymptomatic patients constitute a relatively high percentage (16%). Presence of ascites usually prevents the development of a rigid abdomen by separating the visceral from the parietal peritoneal surfaces [14].

A positive bacterial culture is obtained in the minority of the patients with SBP and results are delayed for several days [7]. In the present study 70% of SBP patients had negative bacterial culture. Currently the diagnosis of SBP is based on PMN count but this test sometimes lack sensitivity and can delay the diagnosis [15]. A delay in antibiotic therapy entails a high mortality rate. On the basis of these considerations, considerable efforts have been made in the recent years to develop an alternative test for more rapid diagnosis.

Lactoferrin is an iron- binding protein contained in PMNs that is released on degranulation [16]. Titers of lactoferrin correlate with absolute neutrophil count in blood samples, and with the presence of neutrocytic inflammation in body fluid such as sputum samples [17]. Similar to the proposed utility of lactoferrin in the diagnosis of SBP, measurements of fecal lactoferrin was evaluated as a mean to diagnose inflammatory diarrhea in a community setting where cell lysis and specimen transport might result in false negative results [10]. Lactoferrin also has been shown to be remarkably stable and resistant to degradation when left at room temperature for extended periods of time. This property makes this marker attractive for clinical use [18].

In this study, ascitic fluid lactoferrin was assessed in cirrhotic ascitic patients with or without SBP to evaluate its role in the diagnosis of SBP. The mean ascitic fluid lactoferrin level was significantly elevated in SBP patients. Our results confirm the previous results reported by Parsi et al. [16]. The elevation of lactoferrin level in patients with SBP could be explained as had been described that lactoferrin is a major component of specific granules of human PMN leukocytes to be actively secreted by these cells into the environment in response to inflammation, bacterial infection and cytokine stimulation [19]. Ascitic fluid lactoferrin was significantly correlated with ascetic PMNs count ($r=0.56$, $p<0.001$) in this study and also with LDH A/S ratio($r=0.79$, $p<0.001$) . In SBP bacteremia with subsequent bacterial localization in the ascitic fluid would make the amount of bacterial DNA which stimulates the immunological response more pronounced in the ascitic fluid rather than blood, , this fact explains rising of lactoferrin level in ascitic fluid more than blood [20]. Lactoferrin plays a role in the first line of defense against microbial infections to prevent invading pathogens from utilizing host iron supplies for multiplication [21, 22].

ROC curve analysis identified an optimal ascitic lactoferrin level of 270 ng/ml for diagnose SBP. Ascitic lactoferrin concentration ≥270 ng/ml had 96%sensitivity and 95% specificity, 97.96% positive predictive value, 90.5% negative predictive value, and 95.7% accuracy in diagnosis of SBP. Parsi et al. [16] demonstrated 95.5% sensitivity and 97% specificity at a cutoff 242 ng/ml. The high sensitivity and specificity suggest that lactoferrin could act as a surrogate marker for PMN count in ascitic fluid in diagnosis of SBP.

The results concerning ascitic fluid lactoferrin in SBP represent an interesting and promising area of investigation, which could determine the further optimization of SBP management and further improvement in its prognosis. An early start of antibiotic therapy is important for the successful treatments of SBP. The development of bedside test that can diagnose SBP rapidly might facilitate patient selection for further diagnostic tests or admission to hospital and improve the cost effectiveness of SBP diagnosis. Qualitative and rapid tests are already commercially available for bedside measurements of lactoferrin concentration in stool. . In those centers in which PMN count and lactoferrin in ascitic fluid can not be measured, a reagent strip for leukocyte esterase designed for the testing of urine is a rapid, easy to use, and inexpensive tool for diagnosis of ascitic fluid infection [23]. A study done on two types of reagents, however, concluded that the negative predictive value for strips for be a high mortality

disease is not enough to discard SPB. In terms of the severity of SPB, the rate of false negative results could be considered high [24].It was previously hypothesized that qualitative tests able to detect lactoferrin levels in excess of a predetermined level for bedside diagnosis can easily be developed with limited costs [16].

In conclusion, measurement of ascitic fluid lactoferrin may serve as a rapid and reliable screening tool for SPB in patients with cirrhosis. Further studies are recommended to compare lactoferrin to other possible diagnostic markers.

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