RESEARCH ARTICLE

Diagnostic validity of flow cytometry vs manual counting of polymorphonuclear leukocytes in spontaneous bacterial peritonitis

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Background: Spontaneous bacterial peritonitis (SBP) is frequently occurring infection among patients with liver cirrhosis, defined by polymorphonuclear (PMN) leukocytic count ≥ 250 cell/mm³ with or without a positive ascitic fluid (AF) bacterial culture. So, this study aimed to investigate the diagnostic value of flow cytometry versus manual counting of ascitic fluid PMNL in cirrhotic patients, with clinical suspicion of SBP.

Methods: A hospital-based cross-sectional study was carried out on 320 cirrhotic patients with clinical suspicion of SBP. Abdominal paracentesis was performed in all cases for microscopic manual and flow cytometry counting of PMNL. Anti-HLA-DR, anti-CD15, anti-CD16, and anti-CD45 monoclonal antibodies were used for flow cytometry method.

Results: Flow cytometric PMNL count had 100% sensitivity and specificity, while manual PMNL count had a sensitivity of 65.52% and specificity of 90% with significant difference (P value < .05).

Conclusion: Flow cytometry is more reliable rapid method for PMNL counting, than the manual method that is less accurate and time-consuming in diagnosing clinically suspected SBP.

KEYWORDS

flow cytometry, polymorphonuclear leukocytes, spontaneous bacterial peritonitis

1 | INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequently occurring infection among patients with liver cirrhosis.¹ Its frequency ranges between 10% and 30% among those patients with high mortality rate (>80%) among untreated patients, and the course and the outcome depend on rapid diagnosis and prompt antibiotic therapy.^{2,3}

The gold standard criteria for diagnosis of SBP is the ascitic neutrophilic count, defined by polymorphonuclear (PMN) leukocytic count ≥ 250 cell/mm³ with or without a positive ascitic fluid (AF) bacterial culture.⁴ The presence of positive AF culture is to confirm the diagnosis but not a necessary requirement for initiation of antibiotic therapy,³

as it takes days to weeks and spontaneous recovery occurs to patients with positive culture with normal PMN,⁵ in addition to the frequently occurring culture-negative neutrocytic ascites variant in approximately 30%-50% of patients.⁶

Manual microscopic PMNL counting, the reference method for white blood cell (WBC) differential count, is time and labor intensive, misclassify cells having difficult morphology and inaccurate in patients with low WBC count,⁷ also it cannot be carried on emergency basis and liable to human errors.⁸ This may lead to delay in diagnosis and treatment of such patients with increasing mortality, while systemic use of empirical antibiotics will increase the iatrogenic complications and medical cost.⁹

Various studies support the use of flow cytometric differential counts as a rapid accurate and improved reference method for PMNL and other differential counts.^{7,10} Thus, this study was carried out to compare the diagnostic accuracy of manual versus the flow cytometric PMNL counting in diagnosing patients clinically suspected to have spontaneous bacterial peritonitis.

2 | MATERIALS AND METHODS

2.1 | Enrollment of subjects

This hospital-based cross-sectional study was carried out on 320 patients recruited from Tropical Medicine and Gastroenterology Department and Internal Medicine Department—Division of Gastroenterology, Qena and Assiut University Hospitals—Egypt, after obtaining ethical approval from the University Hospital Ethics Committee, and an informed written consent was taken from every included patient in this study. The study period was from August 1, 2016 to February 1, 2017.

The included patients had the diagnosis of cirrhotic ascites based on clinical, biochemical, and imaging findings,¹¹ with the inclusion criteria of spontaneous bacterial peritonitis,² which need high index of suspicion, in the form of; Local symptoms or signs of peritoneal infection, or sepsis, or unexplained or sudden hepatic encephalopathy, or renal impairment, or develop gastrointestinal bleeding.

2.2 | Exclusion criteria

Patients with secondary bacterial peritonitis, tuberculous, malignant or pancreatic ascites, immunosuppressive therapy, systemic antibiotics therapy within the previous 1 month for any other infections, trauma, surgery, other viral infections except liver disease, and cancer anywhere in the body including liver cancer, all were excluded from the study.

2.3 | Laboratory workup

Routine laboratory investigations have been carried out to all the included patients in the form of complete blood count (using

FIGURE 1 Flow cytometry dot plots of ascitic fluid different cell populations: A, isotypic negative control; B, CD16 + HLA-DR-neutrophils and CD16 + HLA-DR+ macrophages; C, CD15-positive leukocytes. FL1-H, fluorescent light channel for FITC (Fluorescein Isothiocyanate); FL2-H, fluorescent light channel for Phycoerythrin; PerCP, Peridinin-Chlorophyll Protein

TABLE 1 Demographic and clinical data of patients with clinical suspicion of SBP

Variables	Patients' characteristics (n = 320)
Age (y), Mean $\pm$ SD	59.9 $\pm$ 11.2
Males, (N.%)	272 (85%)
Females, (N.%)	48 (15%)
Child-Pugh class B, (N.%)	30 (10%)
Child-Pugh class C, (N.%)	270 (90%)
Presenting clinical manifestations, (N.%) ^a	
Recent onset abdominal pain	270 (84.3%)
Recent onset abdominal tenderness	195 (61%)
Recent onset fever	265 (88.3%)
Hepatic encephalopathy with no detected precipitating factor	75 (25%)
Recent onset renal impairment with no detected precipitating factor	30 (10%)
Ascitic fluid analysis (Mean $\pm$ SD)	
Glucose (mg/dL)	30.05 $\pm$ 9.52 (60-100)
Protein (mg/dL)	854.95 $\pm$ 110.42 (>300)
Lactate dehydrogenase (LDH) U/L	140 $\pm$ 23 (180-500)
Serum/ascitic albumin gradient ratio (SAAG)	$\geq$ 1.1
Liver functions (Mean $\pm$ SD)	
Aspartate transaminase (U/L)	55.1 $\pm$ 26.6 (0-41)
Alanine transaminase (U/L)	75.5 $\pm$ 25.6 (0-41)
Albumin (g/dL)	2.5 $\pm$ 0.9 (3.2-4.8)
Total bilirubin (mg/dL)	4.3 $\pm$ 4.3 (0-1)
Direct bilirubin(mg/dL)	2.9 $\pm$ 0.6 (<0.4)
Total protein (g/dL)	6.13 $\pm$ 6 (6.2-8.6)
Viral etiology of cirrhosis (N.%)	
Positive HCV	283 (88.5)
Positive HBV	22 (3.4)
Mixed	15 (4.7)
Complete blood count parameters, Mean $\pm$ SD	
Hemoglobin (g/dL)	11.2 $\pm$ 1.3 (11.5-17)
White blood cell count ($10^3/\mu$ L)	6.5 $\pm$ 3.7 (3.5-10.0)
Platelet count ($10^3/\mu$ L)	99.1 $\pm$ 72.3 (150-400)
Neutrophils (%)	67.3 $\pm$ 14.3 (40-75)
Reticulocyte (%)	1 $\pm$ 0.4 (<2)
Coagulation profile, Mean $\pm$ SD	
Prothrombin time (s)	22 $\pm$ 5 (10.5-14.3)
Prothrombin concentration (%)	0.4 $\pm$ 0.1 (80-120)
International normalization ratio (INR)	1.9 $\pm$ 0.4 (0.91-1.23)
Serum creatinine (mg/dL), Mean $\pm$ SD	2.2 $\pm$ 1.6 (0.7-1.1)

^aPatient may present by more than one manifestation.

automated hematology analyzer; cell dyne-1800 (Abbott Diagnostics, USA), colorimetric assays of liver functions (Alanine transaminase, aspartate transaminase, albumin, total and direct bilirubin), serum

creatinine, and serum/ascitic albumin gradient ratio (using using Cobas c311 automated chemistry analyzer, Roche Diagnostics, Germany).

Diagnostic paracentesis was performed for all included patients with clinical suspicion of SBP, at the bedside under sterile conditions on presentation, before starting treatment; the ascitic fluid was collected on EDTA tube and analyzed within 3 hours of aspiration. The ascitic fluids samples were centrifuged and underwent chemical analysis (albumin, glucose, total proteins, and lactate dehydrogenase assay using colorimetric methods), and also stained by Giemsa to determine the total and differential leukocytic counts using an optical microscopic manual method.¹²

Ascitic fluid cultures were performed at the time of presentation, inoculated into BACTEC Peds Plus/F blood culture bottle under complete aseptic conditions, and placed in the BACTEC™ 9050 blood culture instrument with 2 hours of collection, and subcultures were carried out in positive cases to identify the causative organism and were considered negative if no growth after 7 days.

Twenty μ L of ascitic fluid from every included patient was stained with 10 μ L of each of fluorescein isothiocyanate (FITC)-conjugated anti-CD15 antibody, phycoerythrin (PE)-conjugated anti-CD16 antibody, peridinin-chlorophyll protein (PerCP) anti-HLA-DR and allophycocyanin (APC), and anti-CD45 antibody (all from Beckman Dickinson; USA) and incubated 15 minutes (dark room temperature). After incubation, RBCS lysing solution 1 mL 1 $\times$ (from Beckman Dickinson; USA) was added and incubated for an additional 10 minutes at room temperature. Cells were centrifuged at 822 g at 4°C for 5 minutes. The pellet was washed twice with phosphate-buffered saline (PBS), and then, they were resuspended in BD Biosciences fluid sheath.

Analysis of fluorescence data on a minimum of 10 000 cells was acquired by multicolor flow cytometry (FACS Calibur, Becton Dickinson, USA) with the use of cell quest pro software (Becton Dickinson). The cutoffs were taken appropriately by the use of isotope control staining to exclude auto-fluorescence and nonspecific fluorescence caused by nonviable cells which may give false positives. The leukocytes (CD45 positive) were discriminated from the nonhematological cells (CD45 negative) by selecting CD45-positive cells with region on forward scatter (FS) and side scatter (SS), and then, leukocyte subpopulations were gated on the base of the expression of CD15, CD16, and HLA-DR. Granulocytes were identified as CD15-positive and HLDR-negative event, and neutrophils as CD16-positive and HLA-DR-negative events. Eosinophils were identified as CD16-negative and HLA-DR-negative events (Figure 1). Macrophage markers were selected according to their contribution to innate immune mechanisms such as antigen presentation (HLA-DR positive) and phagocytosis (CD16 positive).¹³

2.4 | Statistical analysis

The data were tested for normality using the Anderson-Darling test and for homogeneity variances prior to further statistical analysis. Categorical variables were described by number and

FIGURE 2 Comparative flowchart of flow cytometric versus manual counting of ascitic PMNL among cirrhotic patients with clinically suspected SBP

FIGURE 3 Combined receiver-operating characteristic (ROC) curve for flow cytometry counting of ascitic fluid PMNL in diagnosing clinically suspected SBP

percent (N, %), where continuous variables described by mean and standard deviation (Mean, SD). Chi-square test and Fisher exact tests were used to compare between categorical variables, while, t-test (parametric test) and Mann Whitney U and Wilcoxon test (non-parametric tests) were used to compare between continuous variables. Roc curve was used to determine the cutoff value, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy. A two-tailed $P < .05$ was considered statistically significant. All analyses were performed with the IBM SPSS 20.0 software.

3 | RESULTS

The present study includes 320 patients with virally induced-cirrhosis, clinically suspected to have SBP with the mean age 59.9 ± 11.2 years old, 272 (85%) males and 48 (15%) females (Table 1). Hepatitis C viral infection was highly frequent than HBV. Ninety percent of the included cirrhotic patients were child-Pugh class C. Abdominal pain,

fever, and abdominal tenderness were among the most frequent presenting manifestation. The included patients showed elevated liver enzymes and serum bilirubin, neutrophilia with impaired coagulation and renal impairment (Table 1).

Ascitic fluid culture was positive in only 95 patients (30%) and 225(70%) of cases were culture negative; the isolated organisms were *Escherichia coli* (*E. coli*) in 65(68.4%), *Klebsiella* in 15 (15.8%), and *Enterobacter* and *Strep. viridans* in 15 (15.8%) cases.

In twenty patients (6.25%) with the clinical suspicion of having SBP, their manual and flowcytometric ascitic PMNL counts were < 250 cells/mm³ together with negative ascitic fluid culture and so the diagnosis of having SBP was canceled (Figure 2).

The comparative diagnostic accuracy of flow cytometry versus manual counting of ascitic PMNL is presented in Figure (2) and (3), which revealed that flow cytometric PMNL count had a sensitivity of 100% and specificity of 100% at lower cutoff level of 264 cells/mm³, at this level, manual PMNL count was lower than 250 cells/mm³, with confidence interval 0.643-0.798 with a standard error 0.04. Manual PMNL count had a sensitivity of 65.52% and specificity of 90% with significant difference (*P* value < .05).

4 | DISCUSSION

The clinical diagnosis of SBP without abdominal paracentesis is insufficient as the clinical manifestations of SBP may be subtle or of insidious onset and need high index of suspicion.³ Ascitic PMNL count of ≥ 250 cell/mm³, regardless the results of AF culture, is nowadays accepted as the best isolated diagnostic indicator for SBP.¹⁴ In this study, ascitic fluid culture was positive in only 30% of the included patients with the highest frequency of gram-negative organisms, mainly *E. coli*. This was in agreement with Kamani et al¹⁵ who reported that 23.5% of cases with clinical manifestations of spontaneous ascitic fluid infection had positive ascitic fluid cultures mainly 72.7% for gram-negative bacteria, mainly *E. coli* which reported in 61.3% of their included patients.

As the microscopic slide manual PMNL counting is a time-consuming and requires experienced personnel, recently, many proposed validated convenient monoclonal antibodies combinations, were available, for PMNL counting by flow cytometry.¹⁶ So in the present study, we compare the diagnostic accuracy of manual versus the flow cytometer counting of PMNL in SBP, besides comparing the accuracy, flow cytometry is a rapid method take about 30 minutes.

In the present study, flow cytometry PMNL counted as the percentage of neutrophil (CD45-positive, CD15- and CD16-positive, and HLA-DR-negative immunophenotyping), which show higher sensitivity and specificity in combination with the clinical suspicion when compared with the manual method, using the cutoff point at ≥ 250 , in a statistically significant manner.

Although it is well known that flow cytometry testing used routinely in diagnosis of leukemia and lymphoma, the literature research regarding the use of flow cytometry in PMNL counting

in ascitic fluid is seldom. Only single study could be traced in literature as regards the possible use of flow cytometry in counting the ascitic fluid cells, to overcome the difficulty to perform and the slow reporting in ascitic fluid cell counts, and it was consistent with our results.¹⁷

5 | CONCLUSIONS

Based on the overall results of the comparative performance characteristics, flow cytometry is a suitable, easier, accurate, and faster method in ascitic fluid cell counting, helping the diagnosis of SBP when clinically suspected in patients with viral-induced liver cirrhosis.

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