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ORIGINAL ARTICLE

Role of 16s rRNA Gene in Early Diagnosis of Spontaneous Bacterial Peritonitis in Cirrhotic Ascitic Patients

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ABSTRACT

Background: *Ascites is a recognized problem in patients with decompensated liver cirrhosis. Spontaneous bacterial peritonitis (SBP) is a massive problem in patients who suffer from cirrhosis with ascites. Early bacterial detection allows great intervention to stop SBP. 16S ribosomal RNA (rRNA) is a universal gene used to detect different bacteria present within a sample.* ***Objectives:*** *This study aimed to evaluate the efficacy of broad range 16S rRNA gene polymerase chain reaction (PCR) in diagnosis of ascitic fluid (AF) infection.* ***Methodology:*** *Fifty cirrhotic ascitic patients were undergone to full history, clinical examination, laboratory tests including, AF specimens analysis for polymorph nuclear leucocytic (PMN) count, culture for bacteria and PCR- for DNA detection of bacteria.* ***Results:*** *Bacteria were separated from 21 (42%) of samples of ascitic fluid, and they were mainly Gram-negative bacteria. The sensitivities of culture for bacteria and PCR in diagnosing AF infection were 53% and 86% respectively, while the accuracies were 62% and 74% respectively relation with PMNL.* ***Conclusions:*** *Bacterial DNA in AF samples may be another method for diagnosis AF infection rather than bacterial culture and PMN count for early detection and treatment of AF infection.*

INTRODUCTION

Ascitic fluid infection (AFI) is a serious problem in patients suffering from liver cirrhosis and is accompanied with high morbidity and mortality [1].

Most patients with AF infection present with symptoms including, fever, major abdominal pain, coma and gastrointestinal bleeding. Nevertheless, a large numbert of patients are totally asymptomatic [2].

Current laboratory recognition of SBP is defined as polymorphonuclear (PMN) leucocytic count ≥ 250 cells/ml and a positive culture from ascitic fluid from the patient. Unfortunately, the prolonged time (1 to 2 days) of culture limits its use for helping the proper antibiotic selection in acute care settings. Ascitic fluid culture were negative in some patients with clinical manifestations suggestive of SBP and an ascitic PMN number of 250, so-called culture-negative neutrocytic ascites. On contrary, a low ascitic leucocytic count (250) with positive culture can be present in another SBP variant called bacterascites, or monomicrobial non-neutrocytic bacterascites[3].

Early bacterial recognition would allow objective intervention for prohibiting of SBP and treatment with the most appropriate therapy [4].

In contrast to culture-based methodologies, the fast and right recognition of bacteria in samples using molecular approach has helped for the rapid proliferation of the use of such strategies [5].

16S rRNA is a universal gene (highly conserved sequence between different species of bacteria) used to detect different bacterial species within a sample[2].

METHODOLOGY

Patients:

This cross-sectional study was conducted in Benha University and National Liver Institute, Menoufia University over the duration from April 2018 to October 2018 and included fifty patients with cirrhosis of liver and ascites. The study was confirmed by the Research Ethical Committee Menoufia University and an informed consent was obtained from each participant before enrollment in the research. The patients in that study with cirrhosis as detected by clinical methods and by laboratory investigations. Exclusion criteria included the presence of any clinical sign of infection and antibiotic intake within the preceding 2 weeks.

Patients were classified into 2 groups depending on PMN count into:

Group 1(SBP group): it included all cases with ascitic PMN ≥ 250 cells/mm^3 with +ve and –ve culture.

Group 2 (Non SBP group): it included all cases with ascitic PMN < 250 cells/mm^3.

Investigations:

Basic clinical signs, blood, and AF characteristics (including age, sex, blood total leukocytic count (TLC), serum albumin, urea, creatinine, total bilirubin, direct

bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), CRP, ascitic fluid TLC, total polymorphnuclear leukocytes (PMN) and bacterial DNA detection) were obtained and analyzed.

Ascitic fluid culture:

Ascitic fluid (AF) specimens obtained by paracentesis and blood samples were taken at admission under strict aseptic conditions. Ascitic fluid samples taken from each patient were injected into a blood culture bottle then incubated at temprature 37°C for at least 7 days or until the positive signal was detected (according to manufacturer's instructions). Sub-cultures were made on blood agar and MacConkey's agar plates. The remain of the AF specimens and sera were kept at 70°C till DNA extraction was done.

Bacterial DNA detection:

An aliquot of AF from all samples was inoculated in rubber-sealed heparin tubes. DNA from each AF sample was detected by using kit (Qiagen, Germany). Presence of DNA from bacteria was assessed by using universal 16s rRNA primer for bacteria, 5-AGAGTTTGATCATGGCTCAG-3 and 5-ACCGCGACTGCTGCTGGCAC-3, which amplify approximately 540 bp fragment. All PCR mixtures were prepared in a amount of 50 µL containing , 2 µl of template that was added into a reaction mix containing 10 mmol/L Tris buffer (pH 8.3), 50 mmol/L KCl, 1.5mmol/L MgCl2, 200 mol/L of each deoxynucleoside triphosphate, 50 pmol of primers and and 1.25 U Taq polymerase (Qiagen, Germany). The mixtures were placed in a thermocycler (PerkinElmer, Norwalk, Conn). PCR was carried out in 35 cycles, each of a denaturation step at 94°C for 30 sec., a primer-annealing phase at 55°C for 30 sec., and an extension phase at 72°C for 60 sec. PCR results were seen by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under ulta-violet light [1].

Statistical analysis:

Results were gathered, grouped and statistically analyzed by an IBM computer with SPSS software version 20.Two types of analysis were done: a) Descriptive statistics e.g. was done in: Number (No), percentage (%) mean ($\bar{x}$) and standard deviation (SD). b) Analytic statistics e.g. - Student's t-test and Mann Whitney's test. - Chi-square test (χ^2). Any cells less than five, Fischer's Exact test was used. P- value of < 0.05 was statistically significant.

RESULTS

Fifty patients had cirrhosis of liver with ascites were involved in that study with mean age 61.8± 8.4 years. They included 33(66%) males and 17(34%) females and there was no statistical difference between SBP and non-SBP groups regarding to age and sex (Table 1).

For biochemical tests serum albumin is significantly lower with p value (0.019) and CRP is considerably higher with p value (0.001) in SBP than non SBP group. Other serum biochemical tests have no statistically significant differences between the studied groups (Table 2).

According to results of AF PMNL count , patients were separated into 2 main groups, First, included 36 (71%) patients with count ≥ 250/mm^3 (SBP group) and the second included 14 (28%) patients with count <250/mm^3 (Non SBP group) (Table 3).

Nineteen 19 (90.5%) of positive samples for culture were ≥250 while 2 (9.5%) of positive culture samples <250.While, there were 17 (58.6%) of culture negative samples were ≥250 and 12 (41.4%) of culture negative samples were <250 (Table 4).

Bacteria were isolated from 21 out of 50 (42%) ascitic fluid specimens. All positive cultures revealed growth of a single organism. Isolated bacteria included: *E.Coli* was the maximum organism isolated from samples 10 (48.0%),then *Klebsiella pneumoniae* 4 (19.0%), then *Staphylococcus aureus* 4 (19.0%) and lastly *Pseudomonas* 3 (14.0%) (Table 5).

Out of 50 ascitic fluid samples, bactDNA was detected in 39 (78.0%) of samples (Table 5).

Table 1: Demographic data of the studied groups

	SBP group N=36		**Non SBP group N=14**		**Test of significance**	**P value**
Age mean ±SD	61.2±9.2		63.4±6.3		**t** 0.82	0.416 **NS**
Sex	**No**	**%**	**No**	**%**	χ^2	**FE**
Male	24	66.7	9	64.3		
Female	12	33.3	5	35.7	0.03	1.000 **NS**

Table 2: Serum Biochemical tests of the studied groups

	SBP group N = 36	**Non SBP group N = 14**	**Test of significance**	**P value**
Total bilirubin (mg/dl) mean ±SD	4.9±7.2	3.1±4.1	**U** 1.37	0.170 **NS**
Direct bilirubin (mg/dl) mean ±SD	3.5±5.2	1.9±2.9	**U** 1.88	0.060 **NS**
AST (IU/l) mean ±SD	127.3±97.1	95.1±104.6	**U** 1.72	0.086 **NS**
ALT (IU/l) mean ±SD	59.5±48.2	38.1±33.8	**U** 1.37	0.170 **NS**
Albumin (g/dl) mean ±SD	2.2±0.51	2.56±0.56	**t** 2.44	0.019 **S**
Urea (mg/dl) mean ±SD	122.1±78.7	98.0±56.9	**U** 0.89	0.370 **NS**
Creatinine (mg/dl) mean ±SD	1.93±1.2	1.9±0.9	**U** 0.47	0.642 **NS**
CRP (mg/dl) mean ±SD	81.5±48.4	39.3±18.9	**U** 3.19	0.001 **S**

Table 3 Ascitic fluid laboratory finding of the studied groups

	SBP group N = 36	**Non SBP group N = 14**	**Test of significance**	**P value**
TLC mean ±SD	2077.8±2055.5	310.4±63.9	**U** 5.44	<0.001 **HS**
PMNL mean ±SD	1746.3±1985.8	204.7±27.5	**U** 5.45	<0.001 **HS**

Table 4 Ascitic fluid positive and negative cultures regarding PMNL count

	Positive culture N=21		**Negative Culture N=29**		**Test of significance** χ^2	**P-value**
	No	**%**	**No**	**%**		
PMNL count						
≥250	19	90.5	17	58.6	6.13	0.013 **S**
<250	2	9.5	12	41.4		

Table 5 Ascitic fluid culture and PCR data of all studied patients

	All studied patients N = 50	
Culture	**No**	**%**
Positive	21	42.0
Negative	29	58.0
Type of organisms among positives	**N = 21**	
E.coli	10	48.0
Klebsiella	4	19.0
Staph.aureus	4	19.0
Pseudomonas	3	14.0
PCR		
Positive	39	78.0
Negative	11	22.0

DISCUSSION

Cirrhosis of liver strikes huge number of persons all over the world. The most severe complications are ascites and SBP [6]. Early discovery of SBP along with proper antibiotic therapy can be useful for patient's life [7].

This study showed that, there was no statistical difference between SBP and non-SBP groups regarding to age and sex. These results agreed with Such et al. [8] and Metwally et al. [9] who stated that age and sex had no significant difference.

Reginato et al.[10] observed predominance of men over women among the study subjects, with age around 60 years. This pattern was similar to what was observed in other reports, and probably reflects the classical history for patients hepatic diseases who seek emergency care centers due to development of ascites.

Regarding the biochemical tests serum albumin was significantly lower and CRP is considerably higher in SBP than non SBP group. This was in agreement with Khorshed et al.[11] and Boaretti et al.[12] who reported that there was a considerable increase in CRP level in SBP patients on contrast with non SBP patients Other serum biochemical tests have no statistically significant differences between the studied groups.

Saleh et al. [1] reported that large number of laboratory tests involving, albumin, bilirubin and creatinine were significantly elevated ($P < 0.05$) in SBP patients rather than non-SBP patients. On the contrary liver enzymes (AST &ALT) were non statistically significant among groups.

In addition, Metwally et al.[9] reported that variables as serum total bilirubin, AST, creatinine, TLC, platelet count and CRP showed a significant difference among the two groups ($p = 0.001$, 0.006, <0.001, 0.003, <0.001 and <0.001). While, serum albumin and ALT had no significant statistical difference ($p = 0.198$ and 0.136).

On contrast Mostafa et al.[13] reported that there was no statistical difference in data of all serum results except AST result; which was massively higher in group SBP than in non-SBP group.

Regarding TLC and PMNL count of ascitic fluid, there was a highly statistical variation among the studied groups. This agreed with Lutz et al.[14] who reported that patients suffering from SBP had the highest leukocyte and PMNL counts.

There was non obvious variation between the studied groups regarding AF PMNL as reported by Mostafa et al.[13]

In our study ascitic fluid bacterial culture showed massive difference between positive and negative culture samples regarding AF PMNL count. Nineteen 19 (90.5%) of culture positive samples PMNL count were ≥250 while 2 (9.5%) of positive culture samples had a count <250. While, there were 17 (58.6%) of culture negative samples were ≥250 PMNL count and 12 (41.4%) of culture negative samples were <250 PMNL count. Also, AF culture showed sensitivity 53%, specificity 86%, PPV 90%, NPV 41% and accuracy 62% for SBP diagnosis.

In the present work, twenty one (42.0%) of ascitic samples were positive for bacterial culture. *E.Coli* was the commonnest organism recovered from samples (10, 48.0%), followed by *Klebsiella pneumoniae* (4, 19.0%), then *Staph. aureus* (4, 19.0%) and lastly *Pseudomonas* species (3, 14.0%).

Saleh et al.[1] reported that thirty-five (26.9%) ascitic samples were positive for bacterial culture. *E.Coli* was the most isolated organism 20 (57.1%) from samples, then *Klebsiella pneumoniae* 9 (25.7%), then *Staphylococcus aureus* 4 (11.4%) and lastly *Staphylococcus epidermidis* 2 (5.7%). Also, it was reported that pathogen identification with AF culture succeeded in 35/130 (26.9%) samples of suspected SBP with low sensitivity and accuracy (31.5% & 41.5% respectively) which is comparable with other prospective and retrospective studies that reported culture-positivity in 34-39%. High rates of culture negativity suggest that such methods are poor in characterizing SBP.

In addition, Sajjad et al.[15] reported that most common group of bacteria isolated in their research was Gram negative bacilli, followed by Gram positive cocci. Amongst the Gram negative bacilli, *E. coli* (59.25%) was the commonest bacteria followed by *Pseudomonas* spp. (22.22%) and *Klebsiella pneumoniae* (11.11%). Amongst the Gram positive cocci, *Staphylococci* and *Streptococci* were 3.70% each.

Regarding PCR analysis of AF for recovery of 16S rRNA gene it was so high in SBP than non SBP group where 39 (78.0%) of samples were positive and with sensitivity 86%, specificity 43%, PPV 79%, NPV 55% and accuracy 74% for SBP diagnosis.

These results were agreed with Malli et al.[7] who reported that the application of 16S rRNA PCR directly in AF showed high specificity (100%) but low sensitivity (25%).

In addition, Saleh et al.[1] found that PCR distinguished DNA from bacteria in their studied AF samples from all positive culture cases (100%) and from (56.8%) of culture negative cases with overall sensitivity and accuracy of 80.1% and 83% respectively.

Rogers et al.[16] showed that molecular results could help in rapid diagnosis of the bacterial types of AF, so rapid and goaled antibiotic intervention. Hardick et al.[17] and Gohar et al. [18] also reported that 16S PCR can be useful in the diagnostic methods for clinicians for identification of organisms responsible for SBP.

CONCLUSION

PCR detection of DNA for bacteria in AF samples of patients accompanied by ascites and cirrhosis can be an alternative way for detection of AF infection earlier than culture of bacteria and PMN count for right treatment of AF infection, aiding in diagnosis of culture negative cases which represent large number of patients that we meet in our routine practice. So, there is a benefit from adding the diagnostic tools of AF infection in patients suffering from cirrhosis accompanied by ascitis.

Conflicts of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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