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Study of Rapid Diagnosis of Spontaneous Bacterial Peritonitis in Cirrhotic Ascetic Patients by Using 16S Ribosomal RNA Gene PCR

Hoda Abdeen Ibrahim[1], Soha Esmat Khorshid[2], Tahia Mohamed Ahmed Mohamed[1], Dalia Hani Mohamed Sayed[1], Thoraya Hosny[1]

Departments of [1]Clinical Pathology, and [2]Tropical Medicine, Faculty of Medicine – Zagazig University, Egypt

***Corresponding author:** Dalia Hani Mohamed Sayed, **Mobile:** (+20) 01002383815, **E-Mail:** daliahani94@gmail.com

ABSTRACT

Background: In cirrhosis, spontaneous bacterial peritonitis (SBP) is the most frequent infection . Rapid and precise identification of bacteria in clinical and scientific settings has been greatly aided by the 16S rRNA gene.

Objective: Assessment of role of 16S rRNA gene in diagnosing SBP among cirrhotic ascitic cases.

Patients and methods: our study was done on 60 adults cirrhotic ascitic patients, classified to 2 groups: Group I (SBP group ═ 38): involved all cases with ascitic fluid PMNL ≥ 250 cells/mm3, Group II (Non SBP group ═ 22): involved all cases with ascitic fluid PMNL < 250 cells/mm3. Ascitic fluid (AF) examination, bacterial culture and polymerase chain reaction (PCR) for detection of DNA were assessed among all cases. **Results:** Significantly greater levels of CRP were seen in the SBP group in comparison to the non-SBP group. Culture had sensitivity 53.3%, specificity 68.2%, PPV 70.5%, NPV 64.9 % and accuracy 60% for SBP diagnosis. PCR had sensitivity 94.7%, specificity 63.3%, PPV 81.8%, NPV 87.5% and accuracy 83.33% for SBP diagnosis

Conclusion: Rapid and precise identification of AF infection is crucial for successful therapy, and polymerase chain reaction (PCR) detection of the 16s rRNA gene in ascitic fluid demonstrates this.

Keywords: Spontaneous bacterial peritonitis, PCR, 16s ribosomal RNA gene.

INTRODUCTION

The Greek words kirrhos, meaning "orange" or "tawny," and osis, meaning "condition," combine to form the medical term cirrhosis. Defined as a diffuse process characterized with fibrosis and nodule formation, cirrhosis [(1)].

Spontaneous bacterial peritonitis is the most common infection among cirrhosis patients (SBP). There is no focal point of inflammation within the abdomen, and no outward signs of infection (such as an intestinal perforation or an abscess), we refer to this as a "spontaneous" rupture of the abdominal wall. Nine percent of cirrhotic patients in hospitals develop SBP, which accounts for a quarter of all infections [(2)].

Only with a paracentesis can the PMN count in the ascitic fluid be determined, which is necessary for a diagnosis of SBP. In the absence of an intra-abdominal and surgically curable source of infection, a PMN count in the ascites of >250 cells/mm3 is indicative of SBP [(3)].

The discovery of the 16S rRNA gene was pivotal in the rapid and accurate identification of bacteria for medical diagnosis and scientific study. Common uses include determining the identity of unknown bacterial isolates from environmental, veterinary, and clinical samples [(4)].

Molecular techniques provide more advantages as their detection through the specimens without pure isolate in addition to nonculturable or nonviable bacteria. For this reason, the 16S rRNA gene has been found useful for the quick detection of bacteremia, culture-negative infections, and infections caused by non-culturable bacteria [(5)].

The study objective was evaluation of regular culture for SBP diagnosis and assessment of the usefulness of the 16S rRNA gene in cirrhotic ascitic patients.

PATIENTS AND METHODS

In a cross-sectional study that was conducted at Clinical Pathology and Tropical Departments Zagazig University on a total of 60 consecutive cirrhotic ascitic patients were recruited in the study. They included 31(51.7%) males and 29(48.3%) females.

Two groups of patients were established based on their PMNL count in ascitic fluid (AF):

Group I (SBP group): involved 38(63.3%) patients with ascitic PMNL count ≥ 250 cells/mm^3 with positive and negative culture

Group II (Non SBP group): consisted of 22(36.7%) cases with ascitic PMNL count <250 cells/mm^3

Inclusion criteria:

1. Cirrhotic ascetic patients with SBP admitted to Zagazig University. Clinical, laboratory, and imaging findings were used to make the diagnosis of cirrhosis.
2. When the neutrophil count in the ascitic fluid is more than 250 cells/mm^3, a diagnosis of SBP is made.

Exclusion criteria:

1. Patients with an unrelated elevated leucocytic count in their ascitic fluid (i.e., bloody ascites or pancreatitis)
2. Upper gastrointestinal bleeding
3. Patients with any clinical sign of infection
4. Non cirrhotic ascites
5. Secondary peritonitis
6. Patients who intake antibiotics within last 2 weeks.

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All cases were subjected to:

1. Full history taking with giving special account to inclusion and exclusion criteria with special care for Manifestations of SBP as (fever, abdominal pain or encephalopathy, etc.)
2. Complete clinical examination to detect manifestations of SBP
3. Abdominal ultrasound
4. Laboratory tests including:
   - Complete blood count (CBC).
   - Blood tests for liver function, including total bilirubin, aspartate transaminase, alanine transaminase, and serum albumin.
   - Kidney function tests including creatinine, urea.
   - Prothrombin time.
   - C-reactive protein (CRP).
5. Ascitic fluid examination count of polymorphonuclear leukocytes (PMNL), bacterial culture, and polymerase chain reaction for 16s rRNA gene detection.

Bacterial DNA detection:

Each sample was used to inoculate heparin tubes with a little amount of AF. DNA was extracted from each AF sample using a commercial kit. A universal bacterial 16S rRNA primer was used to detect bacterial DNA, 5- AGAGTTTGATCATGGCTCAG-3 and 5-CCGCGACTGCTGCTGGCAC-3, They amplify fragments with an average of 540 base pairs.

PCR reactions were set up in thermocyclers using a solution comprising 50 pmol of primers, 1.25 U of Taq polymerase, 200 mol/L of each deoxynucleoside triphosphate, 50 mmol of KCl, 1.5 mmol of MgCl2, 10 mmol/L of Tris buffer (pH 8.3), and 50 mmol of KCl. **(PerkinElmer, Norwalk, Conn).**

Three minutes were spent at 94 degrees Celsius for denaturation, 30 seconds at 55 degrees Celsius for primer annealing, and 60 seconds at 72 degrees Celsius for extension in a total of 35 PCR cycles. We were able to examine the PCR outcomes by running a sample through an electrophoresis gel made of 2% agarose, staining it with ethidium bromide, and viewing it under UV light [(6)].

Ethical approval

An approval of the study was obtained from Zagazig University Academic and Ethical Committee. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Statistical Analysis

The Statistical Product for the Social Services (SPSS) version 20 was used for the analysis (SPSS). The range of values, from minimum to maximum, as well as the central and quartile values, were employed to describe numerical information. Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) to calculate difference between two or more groups of qualitative variables. Quantitative data were expressed as mean ± SD (Standard deviation). Independent samples t-test was used to compare between two independent groups of normally distributed variables (parametric data). P value < 0.05 was considered significant.

RESULTS

Age and gender did not differ significantly between the SBP and non-SBP groups in this research of 60 patients with cirrhosis of the liver and ascites (mean age: 62± 7 years), (**Table1**).

Table (1): Patient characters:

Variables		SBP group		Non SBP group		Test	P value
		N=38	%	N=22	%		
Age		61.97±5.89		62.55±7.80		-0.321*	0.749
Sex	**Female**	20	52.6	9	40.9	0.767**	0.381
	Male	18	47.4	13	59.1		

(*independent sample t test)(** Chi-square test)

Complete blood count values did not differ significantly between SBP and non-SBP groups (**table 2**).

Table (2): Complete blood count of cases:

	SBP group	Non SBP group	Test	P value
	Mean± SD	Mean± SD		
Hb (g/dL)	10.34±1.16	10.10±1.03	0.793*	0.431
WBCs (mcL)	11217.55±2635.71	11840.91±2551.55	-0.683**	0.495
Neutrophils	9292.11±2315.61	9431.82±2351.17	-0.377**	0.706
Lymphocytes	1431.58±350.13	1455.91±348.45	-0.555**	0.579
Platelets (mcL)	120.03±25.39	120.32±19.59	-0.046*	0.963

(*independent sample t test)(** Man Whitney test)

In (**table 3**) the SBP group has far greater CRP than the other groups. No statistically significant differences were found using other serum biochemical tests.

Table (3): Liver and kidney function test between Study groups:

Variables	SBP group(38)	Non SBP group(22)	Test	P value
	Mean± SD	Mean± SD		
Total bilirubin (µmol/L)	3.08±0.75	3.13±0.71	-0.246**	0.806
Direct bilirubin (µmol/L)	2.56±0.51	2.54±0.50	-0.299**	0.765
AST (U/L)	125.05±18.62	123.95±13.56	0.437 *	0.664
ALT (U/L)	58.84±14.2	307.68±74.11	-2.588**	0.010
Albumin (g/dL)	2.42±0.35	2.48±0.60	-1.303**	0.193
Urea (mg/dL)	116.74±11.24	114.64±10.61	-0.783**	0.433
Creatinine (mg/dl)	0.86±0.21	0.87±0.20	-0.85**	0.933
CRP (mg/L)	46.24±10.82	24.34±5.83	6.07*	<0.001

(*independent sample t test)(** Man Whitney test)

Cultural data showed no discernible differences between the groups (**table 4**).

Table (4): Culture data of cases

		SBP group		Non SBP group		X^2	P value
		n=38	%	n=22	%		
Culture	Negative	17	44.7	15	68.2	3.077	0.079
	Positive	21	55.3	7	31.8		

Culture had sensitivity 55.3%, specificity 68.2%, PPV 70.5%, NPV 64.9% and accuracy 60% for SBP diagnosis (**table 5**).

Table (5): Sensitivity, specificity and accuracy of culture test.

Sensitivity	Specificity	PPV	NPV	Accuracy
55.3%	68.2%	75.0%	64.9%	60.0%

Out of 60 ascitic fluid samples 16 samples were negative (2 in SBP, 14 in non SBP), 44 samples were positive (36 in SBP, 8 in non SBP) so a large statistically significant difference was found between the groups that were compared (**table 6).**

Table (6): Results of PCR for the cases:

Variable		SBP group		Non SBP group		X^2	P value
		n=38	%	n=22	%		
PCR	**Negative**	2	5.3	14	63.6	24.278	<0.001
	Positive	36	94.7	8	36.4		

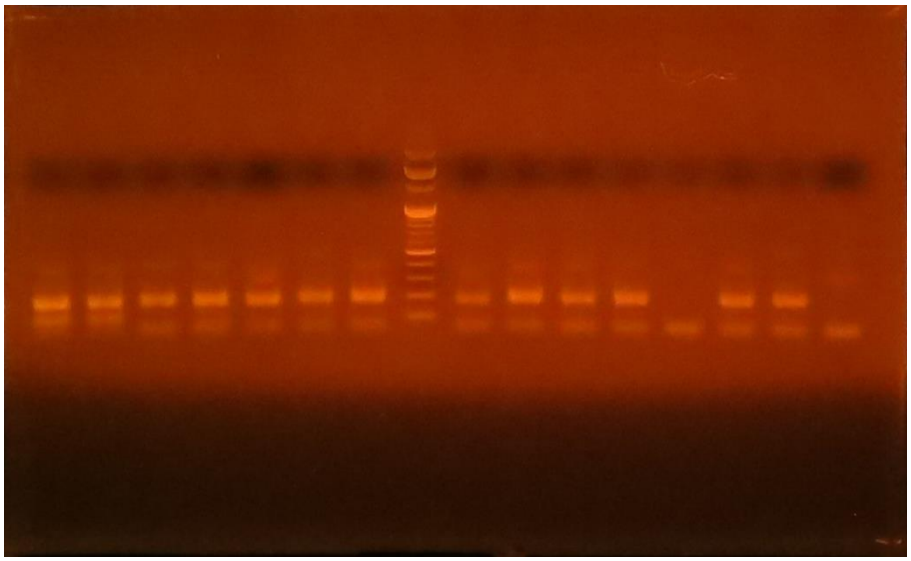

Figure (1): Gel electrophoresis showing PCR positive and negative result.

PCR had sensitivity 94.7%, specificity 63.3%, PPV 81.8%, NPV 87.5% and accuracy 83.33% for SBP diagnosis (**table 7**).

Table (7): Sensitivity, specificity and accuracy of PCR test.

Sensitivity	Specificity	PPV	NPV	Accuracy
94.7%	63.3%	81.8%	87.5%	83.33%

In **table 8** shows that in SBP there were 21 culture positive samples and 36 PCR positive samples while in Non-SBP there were 7 culture positive samples and 8 PCR positive samples.

Table (8): The results of culture and PCR in patients' groups

Patients' group	**Culture**		**PCR**	
	+ve	-ve	+ve	-ve
SBP(No=38)	21	17	36	2
Non-SBP(No=22)	7	15	8	14

DISCUSSION

In order to better diagnose SBP in cirrhotic ascitic patients, this study compared the performance of conventional culture with the 16s rRNA gene. Age and sex did not differ significantly between the SBP and non-SBP groups, as shown by the results of the present study. These findings corroborated those of **Such and colleagues** [7], **Kim and colleagues** [8], and **Yasser and colleagues** [9], who didn't discover any age- or gender-related differences in results.

When comparing CBC indices, there was no discernible difference between the groups. **Gálvez-Martínez *et al.*** [10] reported that Leukocyte count, as well as MCV and MCH levels, were all higher in SBP patients. **Elkafoury *et al.*** [11] showed that Hemoglobin and platelet counts were considerably decreased (P0.001) in the majority of patients based on their hematological testing. There were also significantly more reports of elevated white blood cell count in the SBP group, which is an indicative of the body's inflammatory reaction.

In terms of biochemical tests, CRP were significantly higher in SBP group. This was agreed with **Khorshed and colleagues** [12] and **Boaretti and colleagues** [13] who reported C-reactive protein levels were significantly higher in the SBP group compared to the non-SBP group. Other serum biochemical assays showed no statistically significant differences between the groups.

Saleh and colleagues [6] found that higher albumin, bilirubin, creatinine, platelet, and white blood cell counts were seen in the SBP group when compared to the non-SBP group (P0.05). However, the levels of the two major liver enzymes (alanine aminotransferase and aspartate aminotransferase) did not differ in a statistically significant way.

Heikl and colleagues [14] found that serum total leukocyte count, platelet count, creatinine, urea, total bilirubin, direct bilirubin, all showed statistically significant differences between the SBP and non-SBP groups (P0.001, 0.021, 0.004, 0.002, 0.001, 0.002, and 0.006 respectively). However, there was no statistically significant difference in hemoglobin, alanine aminotransferase, aspartate aminotransferase, or serum albumin when comparing the two groups.

Mostafa and colleagues [15] found that except for a significantly higher AST in the SBP group, no other serum analytical values differed significantly between the SBP and non-SBP groups at the statistically significant level.

When comparing the SBP group with the non-SBP group, the former shows significantly higher values for the bleeding profile variables of prothrombin time (PT), partial thromboplastin time (PTT), and international normalized ratio (INR). This was agreed with **Saleh and colleagues** [6], **Elkafoury and colleagues** [11] and **Metwally and colleagues** [16] who reported that When comparing the bleeding profiles of the SBP and non-SBP groups, a clear distinction can be noticed and attributed this alteration mainly to a defect in the synthesis of coagulation proteins and albumin by hepatocytes in these conditions.

On the other hand, **Lahmer and colleagues** [17] reported that The SBP group did not show a statistically significant difference in INR when compared to the control group.

AF bacterial culture plays an important role in SBP diagnosis. Seventy-five percent of the culture-positive samples from the ascitic fluid were found to have bacterial counts below 250, while 25% of the culture-positive samples had bacterial counts over this threshold. In contrast, 17 (53.1%) of the culture-negative samples were below 250, while 15% (46.9%) were above 250.

Accordingly, ascitic fluid culture showed sensitivity 55.3%, specificity 68.2%, PPV 75%, NPV 64.9% and accuracy 60% for SBP diagnosis.

Saleh and colleagues [6] sampled AF for germs and found that 35 (26.0%) were positive. Only 35 out of 130 (26.1%) samples of possible SBP were positive for the pathogen through AF culture, although the method had a low sensitivity and accuracy, they reported (31.5 percent & 41.5 percent respectively). There is strong

evidence that such approaches are ineffective in identifying SBP due to the prevalence of cultural bias.

While **Amin and colleagues** [18] reported that the traditional (culture-bottle) method of ascitic fluid culture yielded a positive result in only 4 patients while 96 patients had negative cultures, indicating an obviously low rate of culture-positive cases. Culture negative SBP refers to the subset of SBP ascites cases in which bacteria are present but at such a low concentration that a diagnosis cannot be made using standard microbiological culture techniques. Ascites fluid culture and sensitivity data were used to classify patients into two groups: culture-positive (n=4) and culture-negative (n=96). Lab parameters were not significantly different between those with positive and negative culture findings (p-value > 0.05).

On the other side, previous studies as **Runyon and colleagues** [19] and **Ginès and colleagues** [20] High rates of culture positive were recorded, often between 72% and 90%. When comparing culture data, it's important to keep in mind that not all research employ blood culture bottles, therefore there may be variation.

When comparing the SBP and non-SBP groups, 43 out of 44 (73.3%) AF samples were positive for the 16S rRNA gene using PCR.

The findings were consistent with **Malli and colleagues** [4] who reported that DNA amplification techniques were used to successfully treat the issue at hand and multiple investigations focused on the clinical significance of this discovery by **Hardick and colleagues** [21] and **Usui and colleagues** [22].

Rogers and colleagues [23] showed that Rapid characterization of the bacterial content of AF using molecular assays could allow for early and tailored antibiotic treatments. **Hardick and colleagues** [21] found that both broad-based 16S rRNA PCR and conventional PCR for SBP pathogen detection and identification were shown to be valuable diagnostic supplementary techniques for doctors. **Soriano and colleagues** [24] found that a higher inflammatory response and poorer survival were linked to the presence of detectable bacterial DNA in 60% of cirrhotic patients with sterile ascites.

Enomoto and colleagues [25] revealed that cirrhotic patients with ascites, a limited number of bacteria are expected to penetrate the intra-peritoneal cavity by multiple pathways, the 16S rRNA gene has been linked to being amplified in cases of early bacterial translocation identification.

Also, **Mostafa and colleagues** [15] and **Soriano and colleagues** [24] stated that with a 76 percent accuracy rate in detecting ascitic fluid infection (AFI), employing nucleic acid amplification techniques for pathogen identification in ascitic fluid is the gold standard, and may be superior to bacterial culture methods in some circumstances, such as when the pathogen in question is difficult to cultivate or when the patient has completed antibiotic therapy.

Conversely, **Zapater and colleagues** [26] also revealed that it has been commonly established that there is no correlation between the presence of bacterial DNA in non-infectious ascites and the development of SBP.

According to our study, PCR sensitivity was 94.7%, specificity was 63.3%, PPV was 81.8%, NPV was 87.5% and accuracy was 83.33% for SBP diagnosis. When PMNL count was ≥ 250 cells/mm^3, 36 (86.1%) samples were PCR positive and when PMNL count was < 250 cells/mm^3 8 (57.1%) samples were PCR positive. While, when PMNL count was ≥ 250 cells/mm^3 the number of positive culture samples were 19 and negative samples were 17 and when PMNL count was < 250 cells/mm^3 the number of positive culture samples were 2 and negative samples were 12. So, the sensitivity of PCR 94.7% is higher than the sensitivity of culture which was 55.3%.

Our results coincides with **Amin and colleagues** [18] who revealed that their report included a 100% sensitivity rate, an 85% specificity rate, a 100% negative predictive value, and an 84% overall accuracy rate. While, **Malli and colleagues** [4] found that the direct use of 16S rRNA PCR in AF was reported to have 100% specificity but just a 5% sensitivity (25 percent).

Additionally, **Saleh and coworkers** [6] found that 100% of culture-positive and 56% of culture-negative AF samples had bacterial DNA that could be detected by PCR (with an overall sensitivity and accuracy of 80.1 percent and 83.3 percent respectively).

Enomoto and colleagues [25] pointed out that it's not uncommon for a bacterial culture to miss the pathogen altogether. It is generally known that many bacterial infections are caused by bacteria that have up to seven copies of the 16S rRNA gene in their genomes. Therefore, in compared to tests intended for a single copy of the gene, the existence of many copies of a gene can increase the likelihood of identifying even low quantities of pathogens. The 16S rRNA gene can be amplified by using the polymerase chain reaction (PCR), which greatly simplifies the research of many different kinds of bacteria. Polymerase chain reaction (PCR) for the 16S rRNA gene has not been shown to positively identify a causative agent. It is difficult to determine the therapeutic value of this method because the 16S rRNA gene can be amplified in both SBP and non-SBP sterile ascites.

However, **Vieira and colleagues** [27] shown that however, bacterial DNA does not appear to permit differentiating between ascites infection and ascites colonization, making amplification of the 16S rRNA gene the preferred method for diagnosing SBP over culture. **Appenrodt and colleagues** [28] discovered no link between SBP and the presence of bacterial DNA in AF.

However, **Tilburg and colleagues** [29] and **Philipp and colleagues** [30] said that this approach had been severely criticized for its inability to detect

bacterial DNA. To begin, most prior research only involved a small number of patients, and a recent analysis encompassing a sizable population of patients yielded disappointing diagnostic results. Furthermore, there is a possibility that the clinical samples will be exposed to exogenous bacterial DNA due to the reagents used in the DNA extraction processes. Despite PCR's high sensitivity for detecting DNA, it has been shown to have mixed results when it comes to diagnosing SBP patients with ascites and identifying the underlying pathogen (s).

Such and colleagues [7] described how the detection of bacterial DNA in cirrhotic individuals may be indicative of a translocation of bacteria that does not result in an overt infection but has detrimental effects on liver function, the immune response, and blood circulation nonetheless.

Some of the limitations and difficulties that need to be addressed in molecular techniques for detecting bacterial DNA include: contamination, a lack of standardized processes and reagents, and issues and unpredictability in the interpretation of data all contribute to the potential for false positive results.

CONCLUSION

- To sum up, bacterial culture and PMN count may not be the best way to diagnose AF infection in cirrhotic ascitic patients, but polymerase chain reaction detection of the 16S rRNA gene in Ascitic Fluid samples may be a better option.
- PCR has higher sensitivity (94.7%) and better accuracy (83.3%) than culture in diagnosis of SBP cases.
- However, it is advised that the ascitic fluid be tested by polymerase chain reaction as the definitive test for the diagnosis of ascitic fluid infection.

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