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ORIGINAL ARTICLE

The Diagnostic Role of Bacterial DNA in Ascitic Fluid Infection in Patients with Cirrhotic Ascites

[1]Mohammed A. Saleh, [2]Eman M. El-sehsah, [3]Ashraf A. Beheiry, [4]Raghda E. Farag*

[1]Microbiology and Immunology, Faculty of Medicine, Al-Azhar University, New Damietta, Egypt; [2]Microbiology and Immunology, Faculty of Medicine, Mansoura University, Egypt.
[3]Medical Biochemistry, Faculty of Medicine, Al-Azhar University, New Damietta, Egypt.
[4] Tropical Medicine, Faculty of Medicine, Mansoura University, Egypt.

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****Corresponding Author:***
Mohammed A. Saleh, PhD,
[1]Department of Microbiology and Immunology, Faculty of Medicine, Al-Azhar University, New Damietta, Egypt.
Email: md_saleh72@yahoo.comE-mail; Tel: 00201006203062

ABSTRACT

Background: *Patients with cirrhosis and ascites carry a high risk of ascitic fluid (AF) infection. Identification of bacterial DNA in patients with cirrhotic ascites may provide a rapid and accurate tool helping in diagnosis of AF infection when it is compared with culture-based methodology to initiate treatment in such cases.* ***Objectives:*** *This study was aimed to evaluate the efficacy of broad range 16 S ribosomal RNA gene polymerase chain reaction (PCR) in diagnosis of AF infection.* ***Methdology:*** *A total of 130 liver cirrhotic ascetic patients were subjected to thorough history, clinical examination, laboratory investigations including, AF samples analysis for polymorh nuclear leucocytic (PMN) count, bacterial culture and PCR- bacterial DNA detection.* ***Results:*** *The sensitivities of PMN count, culture and PCR in diagnosing AF infection were 73.8%, 31.5% and 80.1% respectively, while the accuracies were 77.6%, 41.5% and 83% respectively.* ***Conclusion:*** *Bacterial DNA in AF samples might be an alternative diagnostic method to AF bacterial culture and PMN count in early diagnosis and promp treatment of AF infection.*

INTRODUCTION

Ascites is the most recognized complication in patients with decompensated liver cirrhosis[1]. Patients with cirrhosis and ascites convey a 10% yearly risk of ascitic fluid (AF) infection[2], which is grouped into five categories in light of AF culture, polymorphnuclear leucocytic (PMN) count and the presence of or absence of a surgical source of infection[3,4], including, spontaneous bacterial peritonitis (SBP), culture-negative neutrocytic ascites (CNNA), Monomicrobial non-neutrocytic bacterascites (MNBA), secondary bacterial peritonitis, polymicrobial bacterascites. The initial three are spontaneous variants[5].

Most patients with AF infection present with symptoms including, fever, diffuse abdominal pain and tenderness, alterd mental status and gastrointestinal bleeding[5]. Nevertheless, a significant proportion of patients are totally asymptomatic[6].

SBP is potentially fatal yet reversible cause of deterioration in patients with advanced cirrhosis, occurrs in 10-25% of hospitalized patients with mortality rate ranges from 20-40%[7,8] . It has been depicted as an AF infection in the absence of any intra-abdominal, surgically treatable source of infection and diagnosed on the basis of PMN count ≥ 250/mm^{3}with positive AF culture[9]. It results from translocation of enteric bacteria across gut wall or lymphatics[10] and only a limited number of intestinal bacteria can efficiently translocate [11].

CNNA (plausible SBP) is considered a variant of SBP in which bacteria are available in a low concentration and hence not recognized with microbiological culture methods[12]. However, in light of the fact that clinical symptoms and mortality amongest SBP and CNNA do not vary, current guidelines suggest antibiotic therapy for both disease entities[10,13].

MNBA occurs when the PMN count is <250 cells/mm3, yet growth of a single organism is distinguished on culture. Some of these patients develop SBP so, it may represent a nearly form of SBP [14]. Since studies demonstrated a fundamentally expanded mortality for bacterascites in the presence of clinical symptoms, antibiotic treatment is recommended for symptomatic bacterascites[15].

The term CNNNA indicates that the PMN count is < 250 cells/mm3 and negative AF culture in a patient with ascites. It acquires its significance from the broadly accepted theory that occult episodes of bacterial translocation preced the development of SBP however the number of microorganisms is low to be identified by culture techniqe[16].

Secondary bacterial peritonitis is due to perforation or inflammation of intra-abdominal organs. It ought to

be suspected in patients with relevant abdominal signs or symptoms, multiple organisms in ascitic cultures, and a very high PMN count and/or high protein concentration in the ascites, as well as those who display an inadequate response to therapy[17].

Polymicrobial bacterascites is diagnosed when AF cultures reveal multiple organisms and PMN count < 250 cells/mm3. It occurs due to accidental puncture of the intestines during paracentesis and is associated with low morbidity[18]. Fortunately, it is uncommon condition, occurring in 0.6% or less of paracentesis [5].

All cirrhotic patients with ascites who are admitted to the hospital should undergo diagnostic paracentesis to remove AF paying little mind to their clinical condition[6].

It is now universally agreed that laboratory diagnostics of SBP should be essentially based on PMN count in the AF[11]. Gram stain is rarely helpful for diagnosing SBP and for the accurate identification of pathogens, because of the low number of microorganisms that are typically found in the infected fluid (i.e., usually 1 bacterium/mL). In this way, it had a sensitivity of 10% and a specificity of 97% for recognizing infection[11]. Furthermore, culture techniques are not successful and show negative outcomes in 40 - 60% of cases with SBP as indicated by the worldwide literature[6]. Culture -based analysis of AF has demonstrated that a wide range of bacterial species can be isolated, mostly including, gram-negative bacteria (*Escherichia coli* and *Klebsiella)* in over 50% of cases. Some cases are induced by gram-positive cocci such as Staphylococci and Enterococci [9].

As opposed to culture-based methodologies, the rapid and precise identification of bacteria in clinical samples utilizing culture-independent molecular methods may provide a rational approach to targeted antibiotic therapy when bacterial infection is suspected[13].

Detecting the presence of bacterial DNA in patients with AF infection can be clinically informative, going about as a surrogate marker of bacterial translocation[19]. Nevertheless, few studies have inspected the value of these molecular techniques in the microbiological diagnosis of SBP in cirrhotic patients. Moreover, these reports enrolled a limited number of patients and results were controversial[12].

Therefore, the main aim of the present study was to figure out whether a broad range PCR using primers targeting universal 16 S ribosomal RNA gene (highly conserved sequence between different species of bacteria) increases the efficacy of other techniques (culture& PMN count) in microbiological diagnosis of spontaneous varieties of AF infection in cirrhotic patients with ascites.

METHODOLOGY

Study design and participants

This comparative cross-sectional study was conducted over a period between February 2015 and July 2016 and included 130 liver cirrhotic patients with ascites who were admitted to Tropical Medicine Department, Mansoura University Hospital. Diagnosis was based on thorough history, clinical examination, standard laboratory and ultrasonographic finding[21]. Only patients with viral hepatitis causes were included. In addition, patients with secondary peritonitis, non cirrhotic causes of ascites, or those receiving antibiotics 10 days prior to hospital admission were excluded. Simultaneously, a paracentesis was performed in all patients under aseptic conditions following usual procedures[22]. AF samples were analysed for PMN count, bacterial culture and PCR- bacterial DNA detection as following:

PMN count:

PMN count was performed in EDTA-anticoagulated AF by means of light microscopy[23].

AF culture

Aerobic & anaerobic culture bottles (EDM) with 10 ml of AF at the bed side. Bottles were incubated at 37°C and inspected for growth for at least 7 days. Subcultures were done on nutrient, blood and MacConkey's agar plates. These plates were incubated aerobically and anaerobically at37°C for 2days. Isolated bacteria were identified by standard microbiological methods [10].

Bacterial DNA detection

An aliquot of AF was inoculated in rubber-sealed heparin tubes**.** DNA from each AF sample was extracted by using commercially available kit (Qiagen, Germany). Presence of bacterial DNA was assessed by using universal bacterial 16s rRNAprimer, 5-AGAGTTTGATCATGGCTCAG-3 and 5-ACCGCGACTGCTGCTGGCAC-3, which amplify approximately 540 bp fragment. All PCR mixtures were prepared in a volume 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 deoxynucleosidetriphosphate, 50 pmol of primers and and 1.25 U Taq polymerase (Promega). 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 step at 55°C for 30 sec., and an extension step at 72°C for 60 sec. PCR products were visualized by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under UV illumination[24].

Statistical analysis

Data entry and analysis were accomplished using the SPSS version 17 and EPI Info version 3.5.1 software's (CDC, Atlanta, Georgia, USA). The results were represented in tabular forms then interpreted. Mean, standard deviation, range, frequency and percentage were used as descriptive statistics. One way ANOVA was used to compare quantitative data. Significance was considered at p value less than 0.05. Sensitivity and accuracy of PMN count, bacterial culture and PCR- bacterial DNA detection as tests used for diagnosis of AF infection were calculated.

Ethical approval

The study protocol was reviewed and approved by the local ethical committee of Mansoura Faculty of Medicine, Mansoura University.

RESULTS

A total of 130 patients (mean age 51.56± 12.7 7 years) with cirrhotic ascites were admitted to Tropical Medicine Department, Mansoura University Hospital during the period of this study. They included 89(68.5%) males and 41(31.5%) females.

According to the results of AF PMN count , patients were divided into 2 main groups, First, included 82 (63.1%) patients with count ≥ 250/mm^3 (neutrocytic ascites) and the second included 41 (36.9%) patients with count <250/mm^3 (non neutrocytic ascites)**.**

Combining results of both AF PMN count and culture, 4 patient groups were revealed as in table 1.

Table 1. Distribution of patients among different groups of AF infection

Groups		*PMN count*	*AF culture*	*No (%)*
Neutrocytic ascites	SBP	≥ 250/mm^3	+	26 (20%)
	CNNA	≥ 250/mm^3	-	56 (43.1%)
Non neutrocytic ascites	MNBA	< 250/mm^3	+	9 (6.9%)
	CNNNA	< 250/mm^3	-	39 (30%)

The baseline demographic, clinical and laboratory characteristics of the study participants are shown in table 2.

Table 2. Demographic , clinical and laboratory finding of the studied patients.

	SBP (n=26)	*CNNA (n=56)*	*MNBA (n=9)*	*CNNNA (n=39)*	*P value*
Age (years)	53.4±5.5	52.3±5.3	52.2±7.1	51.2±4.9	0.4
Gender (male/female)	19(73)/7(72)	37(66.1)/19(33.9)	7(77.8)/2(22.2)	26(66.7)/13(33.3)	0.84
Fever	4(15.4)	8(14.3)	0(0)	1(2.6)	0.14
Abdominal pain	13(50)	27(48.2)	4(44.4)	14(35.9)	0.61
Tender abdomen	4(15.4)	10(17.9)	2(22.8)	6(15.4)	0.95
Encephalopathy	3(11.5)	7(12.5)	0(0)	3(7.7)	0.64
Albumin(g/dl)	2.14±0.2	2.15±0.23	2.46±0.4	2.51±0.3	0.00
Bilirubin(mg/dl)	2±0.49	1.7±0.46	1.42±0.17	1.4±0.16	0.001
AST (u/l)	43.9±7.9	44.6±7.7	42.1±5.9	44.4±9	0.8
ALT (u/l)	40.1±9.1	40±9.4	43.3±10.7	38.38.3	0.4
INR	1.6±0.3	1.5±0.2	1.4±0.18	1.4±0.19	0.013
Creatinine(mg/dl)	1.5±0.2	1.5±0.3	1.3±0.2	1.3±0.1	0.00
WBCs count/ mm^3	4550±1792	4408±1800	2911.7±614	3208.9±1445	0.001
Platelet count/ mm^3	71.6±23.8	55.5±14.6	80.4±22.7	80.4±19.2	0.001
AF PMN count/ mm^3	416.9±172.1	443.7±196.5	99.4±61.7	98.4±58.8	0.001
AF culture positivity	26(100)	0(0)	9(100)	0(0)	0.001
AF bacterial DNA positivity	26(100)	34(60.7)	9(100)	20(51.3)	0.001

Data are mean±SD or frequency(%), INR, international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase. P value < 0.05 significant

Thirty-five (26.9%) AF samples were positive for bacterial culture. *E.Coli* was the commonest organism isolated from 20(57.1%) samples, followed by *Klebsiella pneumoniae* 9 (25.7%), then *Staphylococcus aureus* 4 (11.4%) and lastly *Staphylococcus epidermidis* 2 (5.7%). Detection of bacterial DNA in AF by PCR revealed that 89(68.5%) samples were positive showing the suspected DNA band at 540 bp on electrophoresis (figure1). Correlation between AF culture results, bacterial DNA positive PCR and PMN count are listed in table 3.

Table 3. Culture results, bacterial DNA positive PCR and PMN count in the AF samples studied

PMN count	***Culture***		***PCR***	
	Positive	**Negative**	**Positive**	**Negative**
≥ 250/mm3 (82 samples)	26 (74.2%)	56 (58.9%)	60 (67.4%)	22 (53.7%)
< 250/mm^3 (48 samples)	9 (25.8%)	39 (41.1%)	29 (32.6%)	19 (46.3%)
Total	35 (100%)	95 (100%)	89 (100%)	41 (100%)

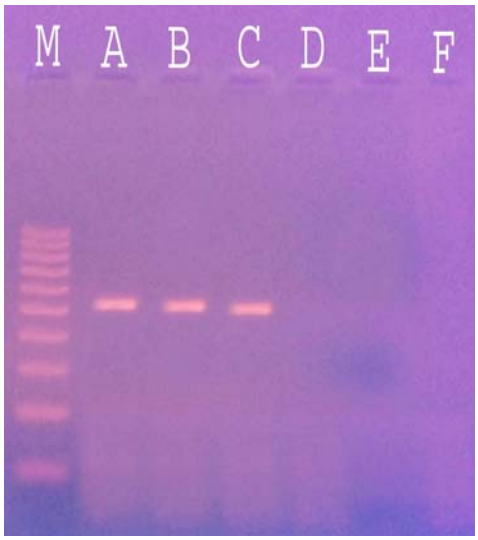

Fig. 1: Agarose gel electrophoresis of bacterial 16S ribosomal RNA gene. Lane M: molecular marker (100 bp DNA ladder marker), Lanes A to C: positive bacterial DNA band at 540 bp of 16 S ribosomal RNA gene. Lanes D to F: negative results.

Out of 130 AF samples examined, 111(85.3%) were diagnosed as AF infection by either one or more of the 3 tests utilized (PMN count, Culture and PCR) and 19(14.7%) were tested as negative. Because of diversity of approaches and absence of true gold standard in diagnosing AFI, calculation of sensitivity & accuracy of each test considering the 111 positive diagnosed cases as reference standard was done. The sensitivities of PMN count, culture and PCR in diagnosing AF infection were 73.8%, 31.5% and 80.1% respectively, while the accuracies were 77.6%, 41.5% and 83% respectively.

DISCUSSION

AF infections are frequent and severe complication in cirrhotic liver patients and have a high morbidity[25]. A high index of suspicion must exist for AF infection in a cirrhotic patient. Given the similarities in presentation between variants of AF infection and inability to clinically distinguish spontaneous from other causes, the diagnosis of SBP should not be empiric[5].

In our study, 4 groups of patients were revealed including, SBP, CNNA, MNBA and CNNNA. The presenting symptoms & signs suggesting AF infection (fever, abdominal pain, tender abdomen & encephalopathy) were manifested among our different patient groups with no statistical significant difference ($P > 0.05$)

Majority of laboratory investigations including, albumin, bilirubin, INR, creatinine, platelets count, WBCs count were significantly elevated ($P < 0.05$) in neutrocytic ascites group rather than non neutrocytic group. On the contrary liver enzymes (AST &ALT) figures were not statistically significant among groups.

Previous studies of Hallak[26], Runyon and Hoefs[27] and Follo et al[28] reported that laboratory evaluation (not including AF analysis) is non specific and not related to SBP per se, but rather, to the underlying hepatic pathology and other accompanying complications and unfortunately not useful in differentiating neutrocytic from non neutrocytic ascites.

In our work, prevalence of SBP was 20% which was correlated well with the results of studies announced by Rimola et al[29] who demonstrated that SBP represents 10-30% of the studied patients . Earlier studies of Marelli et al [30] and Lata et al[31] showed higher prevalence rate of 35.4% and 56.7% respectively. Discrepancy in prevalence rate might be clarified by the way that studies were done on patients with alcoholic cirrhosis who have disturbances of many immunological functions, this consider is uncommon in Egypt . SBP prevalence was lower 5-10% in Wilson et al[32] study. Much lower prevalence of 3.5% reported by Evan et al [33]. These studies carried on outpatients, yet in our study on hospitalized patients and hospitalization may increase the risk of infection.

CNNA in this study was the highest prevailing group representing 43.1% . Marelli et al[30] reported that CNNA occurred in 51.4% of patients in Italy and may not actually represent a different disease entity other than SBP. As needs be, patients should be treated as aggressively as positive culture patients.

In our cohort, MNBA group was the lowest prevalent among AF infection groups representing, 6.9%. Runyon et al[34] results were coordinated with our results with ~8%. Marelli et al[35] revealed that 3.7% of

all ascitic patients was bacterascites. However, Abd EL- Gany et al[1] reported higher prevalence rate of 17%. As high percentage of MNBA patients develop SBP, therefore it may represent a nearly form of SBP [14] as antibiotic treatment is recommended for symptomatic MNBA [15].

Empirical antibiotic treatment for SBP is started when objective evidence of a local inflammatory reaction is present, i.e. AF PMN count ≥250 /mm3 (reliable index of infection), without prior knowledge of the causative organisms or their antibiotic susceptibility[36]. In this study, sensitivity and accuracy of AF PMN count in diagnosing suspicious SBP were 73.8% and 77.6% respectively. In accordance with our results, Enomoto et al[17], Runyon & Antillon[37] and Wiest et al[38] concluded that AF PMN count ≥250 /mm3 is a sensitive method in diagnosis of AF infection and SBP. Nevertheless, reliance on count as a single test that universally agreed conveys the risk of neglection of some cases of AF infection with non neutrocytic ascites and consequent sequalae of bad prognosis and increased mortality due to postponed antibiotic treatment.

In our study, 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%[39,40]. High rates of culture negativity suggest that such methods are poor in characterizing SBP . Plausible clarification for the more negative cultures is a low concentration of bacteria in AF and inadequate culture technique [29] or in some cases of CNNA may represent resolution phase of SBP when the host defence eliminate the organism with out the aid of antibodies but elevated PMN count is still present. On the other side, previous studies reported culture positivity rate to be high mainly ranging between 72% and 90% of cases[41,42] . Discrepancies of culture results may be due to difference in culture techniques as some studies not use blood culture bottles . Additionally, variation in volume of AF samples inoculated ranging from 5 – 10 ml in different studies.

E.Coli was the most common isolated organism among culture positive ascitic patients in this study representing 57.1%. This is in line with prior finding of Abd EL- Gany et al[1] , Bruns et al[13] , Rady et al[43] . Unexpectedly, several reports showed a higher frequency of Gram-positive bacterial infections associated with SBP[44-46].

Despite the fact that AF bacterial DNA detection did not routinely requested in our laboratories, it is available in considerable numbers of laboratories. Application of PCR- based methods for pathogen detection in AF may provide advantages over bacterial culture techniques for microorganisms that hard difficult to cultivate or in patients after antibiotic treatment[13,17]. More than this, PCR can detect a single DNA in samples reporting high degree of sensitivity[24] .

Indeed, in this study, PCR distinguished bacterial DNA in our studied AF samples from all culture positive cases (SBP&MNBA) 35/35(100%) and from 54/95 (56.8%) of culture negative cases (CNNA&CNNNA) with overall sensitivity and accuracy of 80.1% and 83% respectively.

The above mentioned data are in accordance with the previous studies[13, 20,47] which likewise identified DNA in all culture positive cases but, they included small series of patients. Instances of disagreements between cultures and molecular techniques have been accounted for previously[48] . In addition, Such et al [24] and Bruns et al[12] distinguished DNA in 53% of culture negative cases. Some studies[49] reported PCR sensitivities ranging from 75-100%.

Scrutinizing the clinical relevance of bacterial DNA recognition in CNNNA group, it has been interpreted as a reliable indicator of bacterial translocation as occult episodes of bacterial translocation may precede development of SBP [16]. Furthermore, bacterial DNA presence has been shown to predict mortality in patients with CNNNA[50]. Detection of this translocation by PCR is very important in early diagnosis and prompt therapy of suspicious SBP in CNNNA cases[43]. In CNNNA cases in our work, PCR was positive in 51.3% which was correlated well with others[9,24] who identified bacterial DNA in 45% of CNNNA group. Soriano et al[12] found bacterial DNA in a higher percent(60%) while, Serste et al[16] discovered it in only 8/56 (14.2%) of AF samples inspected. Discrepant and controversial results between our data and literatue may be explained by contrasts in analytical sensitivities of various DNA extraction methods and in sequence of primers and difference of patient population (small patients series and antibiotic pre-treatment).

One of the difficulties and challenges of our study which need to be addressed in further investigations is false positive results of PCR due to contamination, what's more, lack of standardization in reagents, methods and results interpretation.

In conclusion, PCR detection of bacterial DNA in AF samples of patients with cirrhotic ascites may be an alternative diagnostic method to AF bacterial culture and PMN count in early diagnosis and promp treatment of AF infection, aiding in diagnosis of culture negative cases (CNNA& CNNNA) which represent large percentage of patients that we meet in our routine practice. So, there is a need to be added to diagnostic tools of AF infection in patients with cirrhotic ascites.

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