ORIGINAL ARTICLE

Combination of vial culture and broad-range PCR for the diagnosis of spontaneous bacterial peritonitis: experience in a Greek tertiary care hospital

E. Malli[1], **N. K. Gatselis**[2], **G. N. Dalekos**[2] **and E. Petinaki**[1]

1)*Department of Microbiology and* 2)*Department of Medicine and Research Laboratory of Internal Medicine*

Abstract

Spontaneous bacterial peritonitis (SBP) is often difficult to diagnose because bacteria in ascites cannot be detected accurately by conventional culture. In this study, we evaluated the use of broad-range 16S rRNA PCR, applied either directly to a total of 32 ascitic fluids (AFs) or to the AF vial cultures, after a long incubation of 14 days; the results were compared with those of AF vial cultures. *Escherichia coli* was isolated in four of 32 AF vial cultures (12.5%). The application of 16S rRNA PCR directly to AF detected only one of the four positive samples (sensitivity 25%, specificity 100%, positive predictive value (PPV) 100%, negative predictive value (NPV) 90.32%). However, the application of 16S rRNA PCR to AF vial cultures after 14 days of incubation correctly identified all the positive samples, including one more that was positive for *Brucella mellitensis* (sensitivity 100%, specificity 80%, PPV 80%, NPV 100%). The elongation of the incubation period of the AF vial cultures, combined with the use of 16S rRNA in negative vials, increases the possibility of identifying the causative agents of SBP and could be applied in the clinical laboratory.

Keywords: 16S rRNA, ascitic fluid, diagnosis, spontaneous bacterial peritonitis

Original Submission: 15 October 2018; **Revised Submission:** 30 November 2018; **Accepted:** 3 December 2018

Article published online: 18 December 2018

Corresponding author: E. Petinaki, Department of Microbiology, University Hospital of Larissa, Larissa, Greece.
E-mail: petinaki@med.uth.gr

Introduction

Spontaneous bacterial peritonitis (SBP) is a frequent and severe complication of individuals with cirrhosis who have ascites [1]. In this group of patients, SBP prevalence ranges from 10% to 30%, and its mortality rate is approximately 20%, despite early diagnosis and treatment [2,3]. The key mechanism of the pathogenesis of SBP is bacterial translocation, defined as the passage of viable gut flora through the intestinal barrier to extra-luminal sites. It has been demonstrated that in cirrhosis there is pronounced impairment of gastrointestinal tract motility. The disturbance of gut flora micro-ecology that follows—in association with changes in the ultrastructure of the gastrointestinal tract and reduced local and humoral immunity—paves the way for the relatively free flow of microorganisms and endotoxins to the mesenteric lymph nodes, subsequent systemic circulation and seeding into ascites [4–6].

Peritoneal infection causes an inflammatory reaction that results in an increased number of polymorphonuclear leucocytes (PMN) in ascitic fluid (AF). SBP is diagnosed on the basis of PMN cell counts $\geq$250 cells/mm^3 in AF, regardless of the result of the ascites culture [7–9]. Although AF culture positivity is not a prerequisite for the diagnosis of SBP, culture must be performed to guide antibiotic therapy [9], because a timely and appropriate antibiotic treatment can improve the clinical outcome significantly. Traditionally, identification of bacteria that cause SBP has depended on culturing, but this normally takes several days. Furthermore, in a high proportion of SBP patients, the AF cultures remain negative, probably due to the relatively low concentration of bacteria or to the presence of fastidious microorganisms that cannot be cultured [1,10]. Runyon *et al.* have demonstrated that inoculation of ascites directly into blood culture bottles (AF vial cultures) at the

https://doi.org/10.1016/j.nmni.2018.12.001

bedside increases the sensitivity of bacterial culture to 90% [9–11]. However, in general practice, ascites culture is negative in approximately 60% of individuals with SBP [1]. The condition of increased PMN count ($\geq$250 cells/mm^3) and negative culture is known as 'culture-negative SBP', and is the most common variant of SBP [12]. Its clinical presentation is similar to that of patients with 'culture-positive SBP' and it should be treated in a similar manner [9].

Molecular assays have been widely used for the detection of microorganisms directly to clinical specimens [13]. However, little international experience exists concerning their introduction in SBP diagnosis. In this study, we evaluated the use of broad-range 16S rRNA PCR applied either directly to AF or to the AF vial cultures after a long incubation (14 days), compared with the traditional approach that includes AF vial culture.

Materials and methods

Ascitic fluid samples analysis

A total of 32 AF samples were collected from individual patients with cirrhosis, who were diagnosed with SPB based on clinical signs, PMN count $\geq$250 cells/mm^3 and absence of intra-abdominal source of infection, and were admitted to the Department of Medicine, University Hospital of Larissa during 2017. Patients who received antibiotic treatment or prophylaxis at admission and/or within the last month were excluded from the study. Samples were obtained under standard aseptic conditions and total cell count, PMN count, Gram stain, total protein and albumin were determined. All AF samples were collected before the initiation of antimicrobial therapy. The protocol was approved by the Ethics Committee of the University Hospital of Larissa, Greece (N 6142).

Ascitic fluid vial cultures, bacteria identification and susceptibility testing

Ten millilitres of each AF was inoculated into blood culture bottles (Bact/ALERT aerobic and anaerobic; Biomérieux, La Balme les Grottes, France) at the patient's bedside. The bottles were incubated for a period of 14 days in a Bact/ALERT 3D (Biomérieux). We note that this prolonged incubation time was chosen by us in order to obtain more positive results.

If there was a positive signal during the incubation, five drops from each bottle were obtained; one for Gram stain and the other four for inoculation onto blood agar plates (aerobically and anaerobically), chocolate agar plate and McConkey agar plate. The bacteria that were isolated were identified and tested for antimicrobial sensitivity using VITEK 2 (Biomérieux).

If no positive signal was detected during the incubation period, the samples were characterized as 'culture-negative' and were further investigated using molecular techniques. Briefly, 100 μL of the positive and negative AF vial cultures' content, at the end of their incubation period, was diluted into 1 mL sterile distilled water and centrifuged at 4000g for 10 min. The supernatant was discarded and 500 μL of the sediment was used for DNA extraction.

Moreover, blood cultures (Bact/ALERT aerobic and anaerobic) were performed for all patients and were inoculated for 5 days, according to our laboratory protocol. In the case of a positive signal, the content of the bottle was examined by Gram stain and sub-cultured onto blood agar plates (aerobically and anaerobically), chocolate agar plates and McConkey agar plates. Isolated bacteria were identified and tested for antimicrobial sensitivity by VITEK 2 as described.

Broad-range 16S rRNA PCR followed by sequence analysis

From each patient, 10 mL of AF was also obtained and centrifuged at 800g for 20 min. After the centrifugation, 9 mL of supernatant was removed and the pellet was mixed by vortexing. A volume of 500 μL was used for DNA extraction using a QIAmp DNA Mini kit (Qiagen, Hilden, Germany), according to the instructions of the manufacturer. The yield and purity of DNA were measured by reading A_{260} and A_{260}/A_{280} in a BioPhotometer (Eppendorf, Hamburg, Germany). In order to assess the success of the DNA extraction and the absence of PCR inhibitors, PCR for the β_2-globulin gene was performed using the primers 5′-GAAGAGCCAAGGACAGGTAC-3′ (forward) and 5′-AACTTCATCCACGTTCACC-3′ (reverse) [13]. In parallel, the 16S rRNA gene was amplified using the universal primers of 5′-AGAGTTTGATCATGGCTCA-3′ and 5′-ACGGCGACT GCTGCTGGCAC-3′ that correspond to a 523-bp 16S rRNA fragment of *Escherichia coli* (positions 8–531) [14]. PCR was carried out through the following cycles: an initial cycle of 95°C for 4 min was followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a final extension period at 72°C for 10 min. Total PCR volume was filtered through QIAquick Spin Columns to remove primers and nucleotides. Purified products were visualized on 1.5% agarose gels stained with ethidium bromide. PCR amplicons were sequenced in both directions in an ABI 3130 genetic analyser and were compared with those submitted to GenBank and EMBL, using the BLAST algorithm [15].

The same protocol was applied in the AF vial cultures. Briefly, a volume of 500 μL of the sediment was used for DNA extraction, while 16S rRNA PCR was performed as described.

Statistical analysis

SPSS version 19.0 (SPSS, Chicago, IL, USA) software was used for data analysis. Specificity, sensitivity, and positive and

negative predictive values were calculated to assess the diagnostic performance of 16S rRNA in AF as well as in AF vial cultures after a 14-day incubation. The reference standard comparator was the AF vial culture.

Results

Among the SBP patients 23 were male and nine were female, with a mean age of 65 ± 8.8 years. Table 1 describes the mean values of various biochemical parameters and PMN count. Macroscopic examination of AFs showed that the colour and the clarity varied between green-brown and cloudy-opalescent, respectively. All AFs had a negative Gram stain.

Of 32 AF vial cultures, four were found to be positive (12.5%); Gram-stain revealed the presence of Gram-negative rods and, based on the VITEK 2 system, the microorganisms were identified as *E. coli*. According to susceptibility tests, all *E. coli* were extended-spectrum β-lactamase producers. The mean time of final culture results was 9 ± 2 days. The remaining 28 vial cultures were negative after a 14-day incubation and were characterized as 'culture-negative'.

Using 16S rRNA PCR directly in all AFs, only one sample was found to be positive (3.1%); sequence analysis of the PCR product demonstrated that the sample was positive for *E. coli*; the result was in concordance with that of the AF vial culture, which was positive for *E. coli*. The time of the final result, including PCR and sequence analysis, was 48 hours. It is interesting that, 16S rRNA gene amplification detected only one out four of 'culture-positive vials' (25%). The application of 16S rRNA PCR in 28 'culture-negative' AF vial cultures revealed that 27 were negative for bacterial DNA presence, and one was positive for *Brucella melitensis* DNA. The four 'culture-positive' AF vials were correctly characterized by the 16S rRNA PCR as *E. coli*-positive, in accordance with their phenotypic identification. Table 2 shows the sensitivity, specificity, and positive and negative predictive values of 16S rRNA PCR.

Regarding the blood cultures of these individuals with SBP, only one of them had a positive blood culture for *B. melitensis*. This individual had a high titre of Wright-Coombs (1/1280) and, as described above, his 'culture-negative' AF vial was positive for *B. melitensis* by PCR.

TABLE 1. Demographic data of the study population and ascitic fluid laboratory results

Parameter	SBP (n = 32)
Age (years), mean ± SD	64.86 ± 8.77
Sex (male/female), *n/n*	23/9
Polymorphonuclear leucocytes, mean ± SD	811.43 ± 99.7/mm^3
Albumin, mean ± SD	0.650 ± 0.75 g/dL
Total protein, mean ± SD	1.85 ± 1.46 g/dL

Discussion

Spontaneous bacterial peritonitis is a severe complication in patients with cirrhosis and ascites. The median colony count in spontaneously infected ascites has been shown to be only one microorganism per mL, which makes it difficult to detect the aetiological agent. The application of DNA amplification methods as a solution to this problem and the clinical relevance of this finding have occupied several studies in the literature [16–21].

Rogers *et al*. showed that molecular assays could provide rapid characterization of the bacterial content of AF, allowing early and targeted antibiotic intervention [17]. Hardick *et al*. have also demonstrated that both broad-based 16S PCR and high-resolution melt analysis provide useful diagnostic adjunctive assays for clinicians in detecting and identifying pathogens responsible for SBP [18]. Soriano *et al*. detected bacterial DNA in AF in 60% of patients with cirrhosis with sterile ascites, and this was associated with an increase in inflammatory response and a worse prognosis [19]. Bruns *et al*. also demonstrated that the application of multiplex PCR identifies microbial organisms in neutrocytic and non-neutrocytic ascites from patients who are at risk for developing SBP [20]. Finally, Such *et al*. detected the presence of bacterial DNA in AF and serum in 9 of 28 patients with cirrhosis and culture negative non-neutrocytic ascites [21].

In contrast, Vieira *et al*. showed that, although the 16S rRNA gene amplification was better than culture to diagnose SBP, bacterial DNA does not seem to allow a distinction between ascites infection and ascites colonization [22]. Appenrodt *et al*. also found no correlation between the detection of bacterial DNA in AF and SBP [23].

TABLE 2. Sensitivity, specificity, positive predictive value and negative predictive value of 16S rRNA PCR applied in ascitic fluid (AF) and in AF vial cultures after 14 days of incubation

Methods	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	PPV	NPV
AF 16S rRNA	1	28	0	3	25	100	100	90.32
AF vial culture 16S rRNA	4	27	1[a]	0	100	96.42	80	100

Abbreviations: FN, false negative; FP, false positive; NPV, negative predictive value; PPV, positive predictive value; TN, true negative; TP, true positive.
[a]The brucellosis case as proved by serology tests and blood culture.

According to the results of our study, using the combination of AF vial culture and 16S rRNA PCR we correctly identified the causative agents in five out of 32 confirmed cases of SBP, including the brucellosis that was verified by serology tests. A prolonged incubation period of more than 5 days, which is the standard protocol in most laboratories, seems to increase the sensitivity of positivity among AF vial cultures. Additionally, the application of 16S rRNA PCR directly in AF showed high specificity (100%) but low sensitivity (25%). Explanation for this discrepancy could be the low concentration of microorganisms, given that these vial cultures gave 'growth signal' after 7 days. Recently, Feng *et al.* applied next-generation sequencing in AFs in patients with cirrhosis and showed that the bacterial 'culture-negative' ascites in these individuals contain much less bacterial DNA than the culture-positive ascites; this finding indicates that the paucity of bacteria, instead of the difficulty of bacterial culture, was possibly the main reason for the negative culture result [24]. Next-generation sequencing technology, by which even trace amounts of bacterial DNA can be detected, could be applied in individuals with SBP to delineate the causative microorganisms.

An interesting finding of our study was the detection by 16S rRNA PCR of *B. melitensis* in one 'culture-negative' AF vial after an incubation of 14 days. The slow growth of the microorganism in association with its low concentration in AF produced a 'culture-negative' vial result. It is known, that the most common microorganisms associated with SBP are Gram-negative bacteria such as *E. coli* and *Klebsiella pneumoniae* [9,25]. Apart from them, there are a few organisms that are considered to have an aetiological role in a small number of cases; this group includes *Yersinia enterocolitica*, *Listeria monocytogenes* and *B. melitensis*. In endemic areas, such our region, clinicians should be aware of the involvement of *B. melitensis* in SBP [22,26,27].

A limitation of our study was the absence of quantitative PCR testing and of the application of other molecular methods (e.g. next-generation sequencing technology) that are more sensitive than the conventional 16S rRNA PCR.

In conclusion, we propose a new protocol for AF vial cultures obtained from individuals with SBP. This protocol includes a long incubation (14 days) of AF vial cultures; if the cultures remain negative, even after this prolonged incubation, we propose the direct application of 16S rRNA PCR in the vials. According to our data, this combination increases the possibility of identifying the causative agent of the SBP.

Conflict of interest

The corresponding author declares no potential conflict of interest on behalf of his co-authors.

References

[1] Rimola A, García-Tsao G, Navasa M, Piddock LJ, Planas R, Bernard B, et al. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. Int Ascites Club J Hepatol 2000;32:142–53.
[2] Garcia-Tsao G. Bacterial infections in cirrhosis: treatment and prophylaxis. J Hepatol 2005;42(Suppl):S85–92.
[3] Piano S, Fasolato S, Salinas F, Romano A, Tonon M, Morando F, et al. The empirical antibiotic treatment of nosocomial spontaneous bacterial peritonitis: results of a randomized, controlled clinical trial. Hepatol Baltim MD 2016;63:1299–309.
[4] Wiest R, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. Hepatol Baltim MD 2005;41:422–33.
[5] Ponziani FR, Zocco MA, Cerrito L, Gasbarrini A, Pompili M. Bacterial translocation in patients with liver cirrhosis: physiology, clinical consequences, and practical implications. Expert Rev Gastroenterol Hepatol 2018;12:641–56.
[6] Guarner C, Soriano G. Bacterial translocation and its consequences in patients with cirrhosis. Eur J Gastroenterol Hepatol 2005;17:27–31.
[7] Runyon BA, AASLD Practice Guidelines Committee. Management of adult patients with ascites due to cirrhosis: an update. Hepatol Baltim MD 2009;49:2087–107.
[8] Hoefs JC, Canawati HN, Sapico FL, Hopkins RR, Weiner J, Montgomerie JZ. Spontaneous bacterial peritonitis. Hepatol Baltim MD 1982;2:399–407.
[9] European Association for the Study of the Liver. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. J Hepatol 2018;69:406–60.
[10] Runyon BA, Canawati HN, Akriviadis EA. Optimization of ascitic fluid culture technique. Gastroenterology 1988;95:1351–5.
[11] Runyon BA, Antillon MR, Akriviadis EA, McHutchison JG. Bedside inoculation of blood culture bottles with ascitic fluid is superior to delayed inoculation in the detection of spontaneous bacterial peritonitis. J Clin Microbiol 1990;28:2811–2.
[12] Runyon BA, Hoefs JC. Culture-negative neutrocytic ascites: a variant of spontaneous bacterial peritonitis. Hepatol Baltim MD 1984;4:1209–11.
[13] Gatselis N, Malli E, Papadamou G, Petinaki E, Dalekos GN. Direct detection of *Cardiobacterium hominis* in serum from a patient with infective endocarditis by broad-range bacterial PCR. J Clin Microbiol 2006;44:669–72.
[14] Rantakokko-Jalava K, Nikkari S, Jalava J, Eerola E, Skurnik M, Meurman O, et al. Direct amplification of rRNA genes in diagnosis of bacterial infections. J Clin Microbiol 2000;38:32–9.
[15] Relman DA. Universal bacterial 16S rRNA amplification and sequencing. In: Persing DH, Smith TF, Tenover FC, White TJ, editors. Diagnostic molecular microbiology: principles and applications. Washington DC: American Society for Microbiology Press; 1993. p. 489–95.
[16] Usui S, Ebinuma H, Chu P-S, Nakamoto N, Yamagishi Y, Saito H, et al. Detection of bacterial DNA by in situ hybridization in patients with decompensated liver cirrhosis. BMC Gastroenterol 2017;17:106.
[17] Rogers GB, Russell LE, Preston PG, Marsh P, Collins JE, Saunders J, et al. Characterisation of bacteria in ascites—reporting the potential of culture-independent, molecular analysis. Eur J Clin Microbiol Infect Dis 2010;29:533–41.
[18] Hardick J, Won H, Jeng K, Hsieh Y-H, Gaydos CA, Rothman RE, et al. Identification of bacterial pathogens in ascitic fluids from patients with suspected spontaneous bacterial peritonitis by use of broad-range PCR (16S PCR) coupled with high-resolution melt analysis. J Clin Microbiol 2012;50:2428–32.

[19] Soriano G, Esparcia O, Montemayor M, Guarner-Argente C, Pericas R, Torras X, et al. Bacterial DNA in the diagnosis of spontaneous bacterial peritonitis. Aliment Pharmacol Ther 2011;33:275–84.
[20] Bruns T, Sachse S, Straube E, Assefa S, Herrmann A, Hagel S, et al. Identification of bacterial DNA in neutrocytic and non-neutrocytic cirrhotic ascites by means of a multiplex polymerase chain reaction. Liver Int 2009;29:1206–14.
[21] Such J, Francés R, Muñoz C, Zapater P, Casellas JA, Cifuentes A, et al. Detection and identification of bacterial DNA in patients with cirrhosis and culture-negative, nonneutrocytic ascites. Hepatol Baltim MD 2002;36:135–41.
[22] Vieira SMG, da Silveira TR, Matte U, Kieling CO, Ferreira CT, Taniguchi A, et al. Amplification of bacterial DNA does not distinguish patients with ascitic fluid infection from those colonized by bacteria. J Pediatr Gastroenterol Nutr 2007;44:603–7.
[23] Appenrodt B, Lehmann LE, Thyssen L, Gentemann M, Rabe C, Molitor E, et al. Is detection of bacterial DNA in ascitic fluid of clinical relevance? Eur J Gastroenterol Hepatol 2010;22:1487–94.
[24] Feng Y, Chen C-L, Chen T-H, Liang Y-H, Chen H-L, Lin C-Y, et al. Application of next-generation sequencing to study ascitic microbiome in cirrhotic patients with or without spontaneous bacterial peritonitis. J Microbiol Immunol Infect Wei Mian Yu Gan Ran ZaZhi 2015;48:504–9.
[25] Oey RC, de Man RA, Erler NS, Verbon A, van Buuren HR. Microbiology and antibiotic susceptibility patterns in spontaneous bacterial peritonitis: a study of two Dutch cohorts at a 10-year interval. United Eur Gastroenterol J 2018;6:614–21.
[26] Makaritsis KP, Liaskos C, Papadamou G, Dalekos GN. Spontaneous bacterial peritonitis: an unusual manifestation of brucellosis in a previous healthy male patient. BMJ Case Rep 2015;2015, bcr2015209387.
[27] Bourantas KL, Christou LG, Dalekos GN, Barbati K, Tsianos EV. A 54-year-old stockbreeder with ascites. Lancet 1997;349:994.