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## CLINICAL STUDY: BACTERIAL DNA IN DIAGNOSIS OF SPONTANEOUS BACTERIAL PERITONITIS

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**CLINICAL STUDY: BACTERIAL DNA IN DIAGNOSIS OF  
SPONTANEOUS BACTERIAL PERITONITIS**

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16  
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53 **KEYWORDS:** ascites, cirrhosis, inflammation, microbiology  
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## SUMMARY

### Background

Despite inoculation into blood culture bottles, ascitic fluid culture is negative in 50% of cases of spontaneous bacterial peritonitis (SBP).

### Aim

To determine whether *16S rDNA* gene detection by real-time PCR and sequencing increases the efficacy of culture in microbiological diagnosis of SBP.

### Methods

We prospectively included 55 consecutive SBP episodes in cirrhotic patients, 20 cirrhotic patients with sterile ascites, and 27 patients with neoplastic ascites. Ascitic fluid was inoculated into blood culture bottles at the bedside and tested for bacterial DNA by real-time PCR and sequencing of *16S rDNA* gene.

### Results

Bacterial DNA was detected in 23/25 (92%) culture positive SBP, 16/30 (53%) culture negative SBP ( $p=0.002$  respect to culture positive SBP), 12/20 (60%) sterile ascites ( $p=0.01$  respect to culture positive SBP) and 0/27 neoplastic ascites ( $p<0.001$  respect to other groups). Sequencing identified to genus or species level 12 culture positive SBP, 6 culture negative SBP and 6 sterile ascites. In the remaining cases with positive PCR, sequencing did not yield a definitive bacterial identification.

### Conclusions

Bacterial DNA was not detected in almost half the culture negative SBP episodes. Methodology used in the present study did not always allow identification of amplified bacterial DNA.

## INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequent and severe complication of cirrhotic patients with ascites. Morbidity and mortality remain relevant even though advances in the management of these patients have improved outcome in recent years.<sup>1-8</sup>

SBP is defined by the peritoneal inflammatory response. An ascitic fluid neutrophil count  $\geq 250/\text{mm}^3$  is currently considered the cut-off for SBP diagnosis.<sup>1</sup> Ascitic fluid culture, however, is not positive in all patients with ascitic fluid neutrophil count  $\geq 250/\text{mm}^3$ . When an alternative cause for the increased ascitic fluid neutrophil count (i.e. bloody ascites, pancreatitis, carcinomatosis or tuberculosis) is absent, these patients are considered to present "culture negative neutrocytic ascites" (CNNA)<sup>1,9</sup> or the more recently introduced designation of "culture negative SBP".<sup>10</sup> Culture negative SBP is considered a variant of SBP in which bacteria are present in a low concentration and therefore not detected with microbiological culture methods.<sup>9,11</sup> In support of this hypothesis, it has been demonstrated that bedside inoculation of large amounts of ascitic fluid (10 and preferably 20 ml) into blood culture bottles increases culture positivity to approximately 70-90% as compared to 30-40% with conventional cultures.<sup>11-13</sup> However, in recent years, even using inoculation of ascitic fluid in blood culture bottles, the rates of bacterial detection in SBP have dropped to less than 50%. This decrease is probably related to the widespread use of antibiotic prophylaxis and to the greater awareness of SBP, leading to diagnosis at earlier stages.<sup>4,14</sup> Moreover, a significant increase in the incidence of SBP caused by multiresistant bacteria has recently been reported to worsen the prognosis of this complication due to the lack of efficacy of current empirical antibiotics.<sup>15,16</sup> This setting makes identification of the responsible bacteria in SBP even more necessary.

Bacterial DNA detection and sequencing is increasingly used in the diagnosis of many infectious diseases. Molecular techniques can detect minimal amounts of bacterial DNA and improve the rates and velocity of bacterial identification.<sup>17-21</sup> Nevertheless, few studies to date have examined the usefulness of these molecular techniques in the microbiological diagnosis of SBP in cirrhotic patients. Furthermore, these reports enrolled a limited number of patients and results were controversial.<sup>22-25</sup>

The aim of the present study was to determine, in a large series of patients, whether broad-range polymerase chain reaction (PCR) using primers targeting the 16S ribosomal DNA (16S

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3 *rDNA*) by real-time PCR (RT-PCR) and sequencing increases the efficacy of culture in the  
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5 microbiological diagnosis of SBP.  
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## 8 9 **PATIENTS AND METHODS**

### 10 **Patients and study design**

11  
12 We prospectively included all cirrhotic patients with SBP diagnosed in our hospital during a 3-  
13 year period (between November 2005 and January 2009). Cirrhosis was diagnosed by biopsy  
14 or based on clinical, analytical and ultrasonographic findings. SBP diagnosis was established  
15 when ascitic fluid neutrophil count was  $\geq 250/\text{mm}^3$  and there was no evidence of an  
16 intraabdominal source of infection.<sup>1,14</sup> Paracentesis was performed in sterile conditions. We  
17 excluded patients with an alternative cause for increased ascitic fluid neutrophil count (i.e.  
18 bloody ascites, pancreatitis, tuberculosis or carcinomatosis) and also those without a complete  
19 microbiological study (i.e. without ascitic fluid inoculation in blood culture bottles). An ascitic fluid  
20 aliquot was stored at  $-80^\circ\text{C}$  until RT-PCR and sequencing of *16S rDNA* gene were performed.  
21 We considered SBP as community-acquired when infection was present at admission and  
22 nosocomial when diagnosis was made after 72 hours of admission or when a previous  
23 neutrophil count during admission was  $< 250/\text{mm}^3$ .<sup>4,14,26</sup> Renal failure at diagnosis of SBP or  
24 prior to SBP resolution was considered when serum creatinine values were above  $133\ \mu\text{mol/l}$ .<sup>27</sup>  
25 Systemic inflammatory response syndrome (SIRS) and septic shock were defined according to  
26 previously established criteria.<sup>2,28</sup>  
27 Patients with SBP were initially treated with intravenous ceftriaxone ( $2\ \text{g/day}$ )<sup>3,29</sup>, unless they  
28 were already receiving this antibiotic at SBP diagnosis. In this case, they were treated with  
29 imipenem.<sup>15,16</sup> Treatment was changed when needed according to the patient's response and  
30 the *in vitro* antibiotic sensitivity. Albumin was administered in patients with urea  $> 11\ \text{mmol/l}$   
31 and/or bilirubin  $> 68\ \mu\text{mol/l}$ .<sup>6,30</sup> SBP was considered resolved when signs and symptoms of  
32 infection disappeared, ascitic fluid neutrophil count was  $< 250/\text{mm}^3$ , and ascitic fluid culture was  
33 negative.<sup>1,10</sup>  
34 We analysed the clinical and analytical characteristics of SBP patients and also in-hospital and  
35 3-month mortality. We compared patients with positive bacterial DNA in ascitic fluid with those  
36 with negative bacterial DNA.  
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3 We also included a group of cirrhotic outpatients with sterile ascites or culture-negative  
4 nonneutrocytic ascites (ascitic fluid neutrophil count  $<250/\text{mm}^3$  and negative culture)  
5  
6 undergoing therapeutic paracentesis in the day-hospital from the Department of  
7  
8 Gastroenterology; and a group of non-cirrhotic outpatients with neoplastic ascites undergoing  
9  
10 therapeutic paracentesis in the day-hospital from the Department of Oncology. In both these  
11  
12 groups we performed the same microbiological studies as those carried out in SBP patients,  
13  
14 including bacterial DNA determination and sequencing.  
15

16  
17 The study was approved by the Research Ethics Committee at Hospital de la Santa Creu i Sant  
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19 Pau and all patients gave consent to be included in the study after receiving appropriate  
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21 information.  
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### 24 25 **Microbiological culture**

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27 **Conventional culture.** We directly inoculated 2-3 drops of uncentrifuged ascitic fluid on  
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29 chocolate agar (BioMérieux, Marcy l'Etoile, France), sheep blood agar (BioMérieux, Marcy  
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31 l'Etoile, France) and Wilkins-Chalgren agar plates (BioMérieux, Marcy l'Etoile, France). Cultures  
32  
33 were incubated in 5%  $\text{CO}_2$  (chocolate agar and sheep blood) or in anaerobic atmosphere  
34  
35 (Wilkins-Chalgren agar) at  $35^\circ\text{C}$  for 4 days and examined daily for visible growth. At the same  
36  
37 time, 0.5-1 ml of uncentrifuged ascitic fluid was inoculated in thioglycolate broth and also  
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39 incubated at  $35^\circ\text{C}$  for 4 days.<sup>13</sup>

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41 **BacT/ALERT blood culture bottles.** In the BacT/ALERT blood culture system (BioMérieux,  
42  
43 Marcy l'Etoile, France), an aerobic and an anaerobic BacT/ALERT blood culture bottle were  
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45 each inoculated with 10 ml of ascitic fluid at the bedside. The bottles were placed in the  
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47 BacT/ALERT instrument and were processed according to the manufacturer's instructions.  
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49 Each bottle was treated independently, and only bottles flagged as positive by the instrument  
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51 were further processed.<sup>13</sup>

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53 Isolated bacteria were identified by standard methods.<sup>31</sup>  
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### 56 57 **RT-PCR and sequencing of 16S rDNA gene (figure 1)**

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59 **DNA extraction.** DNA samples were isolated with QIAamp DNA Blood Mini kit (Qiagen, Hilden,  
60  
Germany) according to manufacturer's instructions with few modifications: 400  $\mu\text{l}$  of ascitic fluid

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3 were centrifuged at 7500 rpm for 10 minutes, resuspended with 180  $\mu$ l of a home-made lysis  
4 solution [Lisozime (20 mg/ml), 20 mM Tris-HCl, pH 8.0, and 1.2% Triton X-100] and incubated at  
5 37°C for 30 minutes. Lastly, proteinase K was added to a final concentration of 20 mg/ml and  
6  
7 incubated at 56°C for 30 minutes and at 95°C for 15 minutes before DNA extraction.  
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11 **RT-PCR.** RT-PCR was performed with a Rotor-Gene 3000 (Corbett Robotics, Australia). Rotor-  
12 Gene has four light-emitting diodes that detect up to four targets in a single amplification  
13 reaction. This characteristic was used to develop specific Taqman® probes to detect all  
14 bacterial DNA with a universal probe (5'-CCACACTGGGACTGAGACACGG-3'- Cy5), *E. coli*  
15 probe (5'-CAGCGGGGAGGAAGGGAGTAAAGT-3'- JOE), and also an internal control probe  
16 (5'-TTAGCCGCTTCCTTCAACTTCACCT-3'-Cy5) corresponding to a soja lecitin gene fragment  
17 (EMBL#K00821) that could be amplified with universal primer adaptors (Biotools, Madrid,  
18 Spain). Probes were selected based on alignments of the *16S rDNA* region with sequences  
19 from the National Center for Biotechnology Information (NCBI) database  
20  
21 (<http://www.ncbi.nlm.nih.gov>). Primers and probes were synthesized at Chipron (Chipron  
22 GmbH, Berlin, Germany) and at Roche Diagnostics (Mannheim, Germany), respectively.  
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RT-PCR was performed in two 25- $\mu$ l reaction mixtures containing 10  $\mu$ l of DNA, 12.5  $\mu$ l of  
Quantitect® Multiplex PCR NoROX (Qiagen, Hilden, Germany), 4  $\mu$ M of primer 27f and 20  $\mu$ M  
of biotinylated primer 519r.<sup>32</sup> DNA from the internal control and 20  $\mu$ M of both *E. coli* and IC  
Taqman® probes were added to one mixture. In the other reaction mixture, 20  $\mu$ M of universal  
probe and 0.5  $\mu$ l of Sau3A1 (1U/ $\mu$ l) were added followed by a digestion at 37°C for 30 minutes  
and 95°C for 5 minutes to inactivate the restriction enzyme. The amplification reaction profile  
included a hot start at 95°C for 15 minutes followed by 35 cycles of 94°C for 60 seconds and  
60°C for 60 seconds. The acquisition of a fluorescent signal was performed at the end of 60°C  
step in all cycles.

To assess the lower limit of detection in ascitic fluid, the approach was performed on 10-fold  
serial dilutions of *Escherichia coli* and *Enterococcus faecalis* ranging from 10<sup>6</sup> to 10 CFU/ml of  
pooled ascitic fluid previously proven to be culture negative. Bacteria used for spiking were  
quantified by agar dilution.

**DNA Sequencing.** Ascitic fluid samples with a universal positive signal and negative for the  
specific *E. coli* probe in the RT-PCR were re-analysed by standard PCR and DNA sequencing.

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3 Standard PCR was performed in a 50- $\mu$ l reaction mixture containing 6  $\mu$ l of DNA from the RT-  
4 PCR product (tube containing the universal probe), 5  $\mu$ l of reaction buffer 10X with  $MgCl_2$   
5 (Expand High Fidelity PCR System, Roche Diagnostics GmbH, Mannheim, Germany), 1  $\mu$ M of  
6 primer 27f and 519r, 10  $\mu$ M of dNTPs and 1.75 U of Taq DNA Polymerase (Expand High Fidelity  
7 PCR System, Roche Diagnostics GmbH, Mannheim, Germany). An initial denaturation at 80°C  
8 for 5 minutes was performed, followed by 28 cycles of 94°C for 45 seconds, 53°C for 1 minute,  
9 and 72°C for 90 seconds, and a final extension at 72°C for 10 minutes. PCR products were  
10 purified with the Exosap-IT PCR System (USB Corporation, Cleveland, Ohio, USA) and  
11 sequenced in both directions using the universal 27f and 519r primers by Macrogen Inc. (Seoul,  
12 Korea). Each sequence was compared with GenBank sequences by using the basic local  
13 alignment scratch tool (BLAST) algorithm.  
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### 27 **Statistical analysis**

28 Statistical analysis was performed by Student's t test and Mann-Whitney test to analyze  
29 quantitative variables, and  $\chi^2$  test and Fisher test for qualitative variables. Results are  
30 expressed as mean $\pm$ standard deviation or frequencies. To analyze survival of SBP patients  
31 according to the presence or absence of bacterial DNA in ascitic fluid, Kaplan-Meier method  
32 with log rank test was used. A p value <0.05 was considered statistically significant.  
33 Calculations were performed with the SPSS Statistical Package (version 17.0, SPSS Inc.,  
34 Chicago, IL).  
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## 45 **RESULTS**

### 46 **Patients characteristics**

47 A total of 69 SBP episodes were diagnosed during the 3-year study period. After excluding  
48 cases with conventional ascitic fluid culture but without culture in blood culture bottles (n=14),  
49 55 episodes in 48 cirrhotic patients were finally included. Of these, 25 had a positive ascitic fluid  
50 culture (45.4%) and 30 had a negative culture (54.5%).  
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56 A group of 20 cirrhotic outpatients with sterile ascites or culture-negative nonneutrocytic ascites  
57 and a group of 27 non-cirrhotic outpatients with neoplastic ascites were also included.  
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3 The characteristics of cirrhotic patients with SBP and sterile ascites are shown in Table 1.

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5 Patients with SBP showed a higher incidence of hepatocellular carcinoma, worse renal function  
6  
7 and higher blood leukocyte and ascitic fluid neutrophil counts than patients with sterile ascites.  
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### 10 11 **16S rDNA gene detection by RT-PCR and sequencing**

12  
13 Tables 2 to 4 show the results of 16S rDNA gene detection by RT-PCR and further DNA  
14  
15 identification by sequencing. The lowest detection limits in ascitic fluid for *E. coli* and *E. faecalis*  
16  
17 were 5900 CFU/ml and 3400 CFU/ml, respectively. Bacterial DNA was detected in 23/25 (92%)  
18  
19 culture positive SBP episodes (Table 2), in 16/30 (53%) culture negative SBP ( $p=0.002$  respect  
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21 to culture positive SBP cases) (Table 3), in 12/20 (60%) sterile ascites ( $p=0.01$  respect to  
22  
23 culture positive SBP and  $p=0.64$  respect to culture negative SBP) (Table 4) and in 0/27 patients  
24  
25 with neoplastic ascites ( $p<0.001$  respect to other groups).  
26

27 RT-PCR or sequencing of amplified fragments identified one microorganism to genus or species  
28  
29 level in 12 cases from the culture positive SBP group (Table 2). The molecular identification  
30  
31 agreed with the culture in 8 of these 12 cases but not in 4. Six of the 30 culture negative SBP  
32  
33 cases gave positive results by molecular techniques to genus or species level (4 *Escherichia*  
34  
35 *coli* and 2 *Streptococcus*) (Table 3). And in 6 of the 20 patients with sterile ascites, positive  
36  
37 results to genus or species level were obtained (all six were *E. coli*) (Table 4). In the remaining  
38  
39 cases with positive RT-PCR, sequencing did not yield a definitive bacterial identification.  
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### 42 43 **Characteristics and outcome of patients with SBP according to presence or absence of** 44 45 **bacterial DNA in ascitic fluid**

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47 Analyzing all SBP episodes ( $n=55$ , Table 5) and comparing positive ( $n=39$ ) and negative  
48  
49 bacterial DNA patients ( $n=16$ ), the former showed more frequent alcoholic etiology, worse liver  
50  
51 function and less frequent treatment with beta-blockers. Moreover, SBP cases with positive  
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53 bacterial DNA showed a trend to a higher inflammatory response (as observed by higher blood  
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55 leukocyte count and incidence of SIRS and a tendency to higher ascitic fluid neutrophil count)  
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57 and more frequent positive ascitic fluid culture.

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59 Renal failure was present at SBP diagnosis in 13/39 (33.3%) patients with positive bacterial  
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DNA and in 3/16 (18.7%) in those with negative bacterial DNA ( $p=0.34$ ). Albumin expansion

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3 was performed in 14/39 (35.8%) patients with positive bacterial DNA and in 2/16 (12.5%) with  
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5 negative bacterial DNA ( $p=0.10$ ). Renal failure at diagnosis of SBP or prior to its resolution was  
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7 observed in 18/39 (46.1%) patients with positive bacterial DNA and in 4/16 (25%) patients with  
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9 negative bacterial DNA ( $p=0.14$ ). The incidence of other complications, such as septic shock  
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11 (6/39, 15.3% vs 0/16, 0%,  $p=0.16$ ), encephalopathy (18/39, 46.1% vs 7/16, 43.7%,  $p=0.87$ ), or  
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13 variceal bleeding (4/39, 10.3% vs 2/16, 12.5%,  $p=1$ ) did not differ significantly between these  
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15 two groups. SBP was resolved in 31/39 (79.4%) and 16/16 (100%), respectively ( $p=0.08$ ). There  
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17 was a trend to higher in-hospital mortality (10/39, 25.6% vs 1/16, 6.2%,  $p=0.14$ ) and 3-month  
18  
19 probability of mortality (31% vs 15%,  $p=0.18$ ) in SBP patients with positive bacterial DNA as  
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21 compared to those with negative bacterial DNA.  
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## 25 DISCUSSION

26  
27 In the present study, RT-PCR detected bacterial DNA in ascitic fluid from most patients with  
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29 culture positive SBP (23/25, 92%). Our results are in agreement with the four previous studies  
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31 including small series of patients with culture positive SBP, that showed positivity of bacterial  
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33 DNA in ascitic fluid in 9/9,<sup>22</sup> 3/4,<sup>23</sup> 5/5,<sup>24</sup> and 4/4<sup>25</sup> patients. Bacterial DNA was negative in two of  
34  
35 our patients. A low percentage of false negative cases has consistently been described in  
36  
37 studies using molecular methods in infections other than SBP, mainly in those caused by gram-  
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39 positive cocci.<sup>18, 33, 34</sup> This has been attributed to a number of factors. Technical difficulties in  
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41 disrupting the cell wall of these organisms may result in failure in DNA extraction.<sup>18,33,34</sup>  
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43 Contamination during sample extraction or processing may lead to a false positive culture in an  
44  
45 otherwise DNA negative sample.<sup>18,25,33,34</sup> Indeed, in our study, cultures grew gram-positive cocci  
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47 in the two cases with positive culture and negative bacterial DNA. Another factor that could also  
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49 contribute is an inoculum effect, as 10 ml of ascitic fluid was inoculated for culture whereas only  
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51 400  $\mu$ l were used for molecular techniques.  
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53 Molecular techniques identified one bacteria in 12 culture positive SBP, eight of which  
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55 correlated with the culture. The lack of agreement in the other 4 cases could be due to  
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57 contamination during sample extraction or processing, or to the presence of DNA from non-  
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59 viable bacteria in addition to the viable bacteria detected in the culture. The latter could be the  
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consequence of previous bacterial translocations, as pointed out below. Again, cases of

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3 disagreement between cultures and molecular techniques have been reported previously.<sup>22,23</sup>

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5 Considering culture negative SBP patients, bacterial DNA was not detected in almost half of  
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7 them (14/30, 46.6%). This finding could perhaps be due to a low DNA concentration in ascitic  
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9 fluid, although an alternative explanation could be that bacteria were not the cause of the  
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11 increased ascitic fluid neutrophil count in these cases. In support of these hypotheses, when we  
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13 analyzed all SBP patients according to the presence or absence of bacterial DNA, we observed  
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15 a trend to a less severe inflammatory response and better outcome in terms of SBP resolution  
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17 and survival in the latter. Data about culture negative SBP are controversial in the four previous  
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19 studies that used molecular techniques in SBP. Bacterial DNA was detected in ascitic fluid in  
20  
21 3/4<sup>25</sup> and 13/13<sup>22</sup> patients with culture negative SBP using conventional PCR, in 1/6 patients  
22  
23 using multiplex PCR<sup>24</sup> and 1/5 with DNA microarray.<sup>23</sup>

24  
25 Molecular techniques can increase the rates and velocity of bacterial identification in  
26  
27 comparison with microbiological cultures.<sup>18,19</sup> They could therefore allow a more suitable choice  
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29 of antibiotic and consequently lead to a better patient outcome.<sup>15,16</sup> In the present study,  
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31 however, the results of bacterial DNA analysis would not have determined a change in the  
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33 antibiotic treatment in any patient. Indeed, one limitation of the present methodology is its  
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35 inability to assess bacterial resistance. Other molecular methods, however, such as DNA  
36  
37 microarrays, may be able to assess such resistances in the future.<sup>18,20</sup>

38  
39 We detected bacterial DNA in ascitic fluid in 60% of patients with sterile ascites. This is  
40  
41 consistent with previous studies by Such et al<sup>35-37</sup> and Viera et al<sup>25</sup> that showed that some  
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43 cirrhotic patients with sterile ascites or culture-negative, nonneutrocytic ascites present bacterial  
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45 DNA in blood and ascitic fluid, and that this presence is associated to an increased  
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47 inflammatory response<sup>22,36</sup> and worse prognosis.<sup>37</sup> The presence of bacterial DNA in non-  
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49 infected cirrhotic patients possibly reflects subclinical bacterial translocation, not leading to overt  
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51 infection but impairing liver function, immune response and hemodynamics.<sup>22,38-40</sup> We found a  
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53 higher percentage of patients with sterile ascites and positive bacterial DNA (60%) than the  
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55 figures previously reported by Such et al. and Vieira et al (approximately 30%).<sup>25,35,37</sup> It should  
56  
57 be noted, however, that our methodology differed from that used in these studies as we  
58  
59 performed RT-PCR with specific and universal Taqman probes instead of conventional PCR. It  
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3 is interesting to point out that bacterial DNA was not detected in any patient with neoplastic  
4 ascites, supporting the reliability of the positive results in cirrhotic patients.

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7 Another interesting finding was that molecular techniques, and specially sequencing, did not  
8  
9 yield a definitive bacterial identification in approximately half the cases in which bacterial DNA  
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11 was detected. These unexpected results could be explained by a suboptimal sequencing  
12  
13 reaction due to a low initial DNA concentration, or by a mix of amplification products  
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15 corresponding to different bacterial species. The latter situation could be attributed to the  
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17 presence of DNA from multiple bacteria in ascitic fluid or polybacterial contamination during  
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19 paracentesis or later processing. Although contamination may have occurred in a few cases it  
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21 would not explain the high percentage of non-conclusive identification, especially taking into  
22  
23 account that bacterial DNA was not detected in 27 ascitic fluid samples from neoplastic non-  
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25 cirrhotic patients.

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27 Accordingly, we hypothesize that the low percentages of bacterial identification among samples  
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29 with positive bacterial DNA could be the consequence of previous polybacterial translocations  
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31 leading to the presence of polybacterial DNA (with or without viable bacteria) in ascitic fluid.  
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33 Indeed, polybacterial cultures have been described in up to 37% of rats with experimental  
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35 cirrhosis and bacterial translocation.<sup>41-44</sup> Further studies with other methodologies able to  
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37 separate and identify polymicrobial DNA could be useful in these cases with multiple  
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39 amplification products. The use of techniques such as denaturing gradient gel electrophoresis  
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41 (DGGE),<sup>45</sup> denaturing high-performance liquid chromatography (DHPLC),<sup>46</sup> bacterial tag  
42  
43 encoded FLX amplicon pyrosequencing (bTEFAP)<sup>47</sup> or web-based application for the analysis  
44  
45 of mixed DNA chromatograms obtained by direct *16S rRNA* gene sequencing<sup>48</sup> may merit  
46  
47 consideration.

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49 When analyzing the limitations of the present study, the low number of patients with SBP and  
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51 negative bacterial DNA could have influenced the lack of statistical differences in some  
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53 parameters when compared to those with positive bacterial DNA. Other issues are that bacterial  
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55 DNA assessment in blood samples<sup>22</sup> or in serial ascitic fluid samples after antibiotic treatment  
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57 initiation, not performed in the present study, could have provided additional data that may have  
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59 improved the interpretation of our results.  
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3 Molecular techniques to detect bacterial DNA still present several limitations and challenges  
4 that need to be addressed, such as false positive results due to contamination, the lack of  
5 standardized methods and reagents, and difficulties and variability in interpretation of results.<sup>17-</sup>  
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9 <sup>19</sup> We conclude that the methodology used in the present study has little usefulness with regard  
10 to bacterial identification in SBP in clinical practice. However, two relevant findings are worthy of  
11 further study. First, bacterial DNA was not detected in almost half of the negative culture SBP  
12 episodes. Second, RT-PCR and sequencing did not always identify amplified bacterial DNA.  
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24  
25

## 26 **STATEMENT OF INTERESTS**

27  
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3 **FIGURE LEGEND**  
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5 Figure 1. Flow-chart showing procedures used in the present study. SBP: spontaneous bacterial  
6 peritonitis; RT-PCR: real-time polymerase chain reaction. *E. coli*: *Escherichia coli*.  
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Table 1. Clinical and analytical characteristics of cirrhotic patients with SBP and sterile ascites.

Data are mean±SD or frequency (%).

	SBP n=55	Sterile ascites n=20	p
Age (yr)	67.6±11.0	64.6±14.3	0.33
Sex (male/female)	31 (56.3%)/24	14 (70%)/6	0.28
Etiology			
Alcohol	18 (32.7%)	10 (50%)	0.17
HCV	24 (43.6%)	5(25%)	0.14
Hepatocellular carcinoma	18 (32.7%)	2 (10%)	0.04
Child-Pugh A/B/C	4/28/23	1/10/9	0.80
Child-Pugh score	9.3±1.9	9.0±1.7	0.51
MELD score	17.2±7.5	14.4±5.3	0.12
Previous SBP	10 (18.1%)	5 (25%)	0.52
Previous ascites	42 (76.3%)	16 (80%)	0.98
Previous variceal bleeding	11 (20%)	7 (35%)	0.22
Previous encephalopathy	18 (32.7%)	6 (30%)	0.82
Sodium (mmol/l)	133.6±5.2	134.0±5.2	0.77
Urea (mmol/l)	13.0±7.9	7.3±4.5	<0.001
Creatinine (μmol/l)	122.8±78.7	88.6±17.9	0.004
Bilirubin (μmol/l)	60.3±66.2	50.0±45.7	0.52
Albumin (g/l)	26.7±4.9	30.4±4.0	0.004
INR	1.56±0.43	1.44±0.28	0.25
Blood leukocyte count (/mm <sup>3</sup> )	10340±8243	6074±2586	0.001
Ascitic fluid neutrophil count (/mm <sup>3</sup> )	5760±10988	26±29	<0.001
Ascitic fluid total protein (g/l)	13.4±6.0	15.4±9.1	0.28

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3 SBP: spontaneous bacterial peritonitis; HCV: hepatitis C virus; MELD: model for end stage liver  
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5 disease score; INR: international normalized ratio.  
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Table 2. Ascitic fluid culture, real-time PCR (RT-PCR) and sequencing results in cases with positive culture SBP.

Nº of cases	Culture	RT-PCR	Sequention
3	<i>Escherichia coli</i>	positive <i>E. coli</i> probe	ND
2	<i>Escherichia coli</i>	positive	<i>Escherichia coli</i>
2	<i>Escherichia coli</i>	positive	undetermined
2	<i>Streptococcus viridans</i>	positive	undetermined
2	<i>Streptococcus viridans</i>	negative	ND
2	<i>Streptococcus pneumoniae</i>	positive	undetermined
1	<i>Klebsiella pneumoniae</i> <i>Citrobacter freundii</i>	positive	undetermined
1	<i>Enterobacter cloacae</i>	positive	<i>Sphingomonas</i>
1	<i>Klebsiella pneumoniae</i>	positive	<i>Escherichia coli</i>
1	<i>Campylobacter jejuni</i>	positive	undetermined
1	<i>Listeria monocytogenes</i>	positive	undetermined
1	<i>Streptococcus pneumoniae</i>	positive	<i>Escherichia coli</i>
1	<i>Streptococcus pneumoniae</i>	positive	<i>Propionobacterium acnes</i>
1	<i>Streptococcus pneumoniae</i>	positive	<i>S. pneumoniae</i>
1	<i>Streptococcus pneumoniae</i>	positive	<i>Streptococcus</i>
1	<i>Streptococcus viridans</i>	positive	<i>Streptococcus</i>
1	<i>Streptococcus anginosus</i>	positive	undetermined
1	<i>Staphylococcus aureus</i>	positive	undetermined

SBP: spontaneous bacterial peritonitis; ND: not done.

Table 3. Ascitic fluid real-time PCR (RT-PCR) and sequencing results in cases with negative culture SBP.

N° of cases	Culture	RT- PCR	Sequention
14	negative	negative	ND
10	negative	positive	undetermined
2	negative	positive <i>E. coli</i> probe	ND
2	negative	positive	<i>Streptococcus</i>
2	negative	positive	<i>Escherichia coli</i>

SBP: spontaneous bacterial peritonitis; ND: not done.

Table 4. Ascitic fluid real-time PCR (RT-PCR) and sequencing results in cases with sterile ascites.

Nº of cases	Culture	RT-PCR	Sequention
8	negative	negative	ND
6	negative	positive	<i>Escherichia coli</i>
6	negative	positive	undetermined

ND: not done.

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Table 5. Clinical and analytical characteristics of patients with SBP and positive or negative ascitic fluid bacterial DNA. Data are mean±SD or frequency (%).

	Negative bacterial DNA n=16	Positive bacterial DNA n=39	p
Age (yr)	69.1±13.7	67.1±9.9	0.54
Sex (male/female)	7 (43.7%)/9	24 (61.5%)/15	0.22
Etiology			
Alcohol	1 (6.2%)	17 (43.5%)	0.007
HCV	11 (68.7%)	13 (33.3%)	0.01
Hepatocellular carcinoma	6 (37.5%)	12 (30.7%)	0.62
Child-Pugh AB/C	13 (81.2%)/3	19 (48.7%)/20	0.02
Child-Pugh score	8.7±1.2	9.6±2.1	0.06
MELD score	14.3±5.7	18.5±7.9	0.06
Previous complications of cirrhosis	14 (87.5%)	30 (76.9%)	0.47
Previous SBP	5 (31.2%)	5 (12.8%)	0.13
Previous ascites	14 (87.5%)	28 (71.7%)	0.30
Previous variceal bleeding	4 (25%)	7 (17.9%)	0.71
Previous encephalopathy	5 (31.2%)	13 (33.3%)	0.88
Previous antibiotic treatment <sup>1</sup>	3 (18.7%)	14 (35.8%)	0.33
Beta-blockers	11 (68.7%)	14 (35.8%)	0.02
Diuretics	11 (68.7%)	27 (69.2%)	1
Nosocomial	3 (18.7%)	14 (35.8%)	0.33
Asymptomatic	5 (31.2%)	10 (25.6%)	0.73
Abdominal pain	6 (37.5%)	13 (33.3%)	0.64
Fever (>38°C)	7 (43.7%)	13 (33.3%)	0.46
Renal failure	3 (18.7%)	13 (33.3%)	0.34

	Negative bacterial DNA n=16	Positive bacterial DNA n=39	p
SIRS	3 (18.7%)	19 (48.7%)	0.03
MAP (mm Hg)	83.4±14.1	81.4±14.8	0.66
Sodium (mmol/l)	135.5±5.1	132.8±5.1	0.09
Urea (mmol/l)	14.0±9.4	12.6±7.3	0.57
Creatinine (μmol/l)	112.3±46.2	127.1±88.9	0.53
Bilirubin (μmol/l)	30.9±18.6	72.4±74.7	0.002
Albumin (g/l)	26.8±4.4	26.7±5.2	0.93
INR	1.36±0.14	1.64±0.48	0.003
Blood leukocyte count (/mm <sup>3</sup> )	6588±2713	11880±9235	0.002
Ascitic fluid neutrophil count (/mm <sup>3</sup> )	2649±4183	6964±12392	0.18
Ascitic fluid neutrophil count ≥500/mm <sup>3</sup>	9 (56.2%)	32 (82%)	0.08
Ascitic fluid total protein (g/l)	14.3±6.6	13.0±5.7	0.48
Positive ascitic fluid culture	2 (12.5%)	23 (58.9%)	0.002

HCV: hepatitis C virus; MELD: model for end stage liver disease score; SIRS: systemic inflammatory response syndrome; MAP: mean arterial pressure; INR: international normalized ratio.

<sup>1</sup> Previous antibiotic treatment consisted of norfloxacin in the 3 patients with negative bacterial DNA. In patients with positive bacterial DNA, previous antibiotic treatment consisted of norfloxacin in 6 patients, amoxicillin/clavulanic acid in 4, ceftriaxone in 3 and cloxacillin in one.

