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ORIGINAL ARTICLE

Presence of multiple bacterial markers in clinical samples might be useful for presumptive diagnosis of infection in cirrhotic patients with culture-negative reports

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Abstract Bacterial infections in cirrhotic patients with ascites are associated with a severe prognosis and an increased risk of death. The microbiological standard tests for the diagnosis of suspected infection, based on culture test of blood and ascitic fluid, are, in many cases (30–40 %), negative, even when patients show symptoms of infection. A multiple culture-independent protocol was applied and evaluated as a diagnostic and prognostic tool for the detection of bacterial infection in cirrhotic patients. Sixty-four culture-negative samples obtained from 34 cirrhotic patients, with PMN < 250 cells/μl of ascitic fluid, were screened for the presence of bacterial DNA, endotoxin, peptidoglycan/β-glucan and microscopically visible bacterial cells. Correlations between the presence of multiple markers and various clinical and laboratory parameters were evaluated. Bacterial DNA was detected in 23 samples collected from 16 patients; a large part of these samples also showed the presence of other bacterial markers, which was associated with a worsening of liver functionality, a higher incidence of infections during the follow-up and a higher mortality rate in our cohort of cirrhotic patients. We believe that the detection of additional bacterial markers in bacterial DNA-positive clinical samples makes the bacterial presence and its clinical significance more realistic and might be useful as early markers of an ongoing bacterial infection and in establishing a clinical prognosis.

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Introduction

Patients with severe liver diseases are particularly susceptible to bacterial infections, with an incidence rate reaching 30 % [1]. In these patients, often presenting cirrhosis and ascites, bacterial infections are associated with a severe prognosis and an increased risk of death [2–4]. The pathogenic mechanism at the basis of the development of spontaneous infections is related to a phenomenon known as bacterial translocation (BT) [5, 6], by which viable enteric bacteria cross the gut wall, reaching the mesenteric lymph nodes, and, from there, the systemic circulation and other locations. The importance of the sequence of events starting from bacterial translocation and leading up to bacterial infection in the cirrhotic patient has been increasingly recognised in recent years [7, 8]. Numerous factors are probably involved in promoting pathological BT in cirrhosis, including the overgrowth of intestinal microbiota, the structural and functional alteration of the intestinal barrier function and the impaired activity of the gut-associated lymphoid tissue (GALT) [9, 10]. These pathophysiological alterations are believed to be associated not only with the translocation of viable bacteria but also with the translocation of bacterial fragments or components such as DNA [2, 11, 12], which can induce a marked inflammatory response of the host immune system, resulting in tissue damage and organ failure [11–13]. The most frequent infections observed in cirrhotic patients are spontaneous bacterial peritonitis and urinary tract infections. Pneumonia, skin and soft tissue infections and sepsis could also occur. The most frequent causative microorganisms of these infections are enteric Gram-negative rods, mainly *Escherichia coli* and Gram-positive cocci [14]. Early diagnosis and treatment of these infections are mandatory when managing patients with cirrhosis and ascites.

Bacterial translocation could be diagnosed by the mesenteric lymph node-positive culture, but this diagnostic approach is quite difficult and rather invasive; therefore, alternative

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approaches to diagnosing BT in humans have been proposed. Typically, microbiological diagnosis of bacterial infection in cirrhotic patients is based on culture test of blood (blood culture) and of ascitic fluid. However, in many cases (30–40 %), these standard tests are negative, even when patients show symptoms of infection [6, 15].

The lack of isolation of the involved aetiological agent might be due to incorrect sampling, inadequate conservation of the specimen, low microbial concentration in the sample and the involvement of hardly growing or non-recoverable bacteria. To overcome this problem, several authors have proposed the detection of bacterial DNA in serum and ascitic fluid (AF) as an alternative method for the diagnosis of BT. The clinical significance of the presence of this bacterial molecule in patient samples has not been completely elucidated, even though it seems to play a role in inflammatory response and could be a predictor of poor prognosis in cirrhotic patients with ascites [2, 12, 16].

In this study, we analysed the possibility of using multiple microbiological markers to early detect the presence of bacteria in steps preceding a fully developed infection in order to establish a presumptive diagnosis of infection in patients with a culture-negative report. Moreover, with the aim of determining the clinical significance and prognostic value of the presence of multiple bacterial indicators in culture-negative clinical samples, possible correlations between microbiological and clinical data were evaluated.

Patients and methods

Patients and clinical samples

A homogeneous group of patients with cirrhosis and ascites was sequentially enrolled in this study and recruited at the Gastroenterology Unit of the Polyclinic Hospital Umberto I in Rome (a tertiary referral centre). The diagnosis of cirrhosis was made after hepatic biopsy or endoscopy when available, or on the basis of clinical and biochemical parameters. The PMN counts in the ascitic fluid were lower than 250 cells/μl in all the patients.

For each subject, demographic and various clinical and biochemical parameters were recorded. The severity of chronic liver diseases was evaluated using the following indexes: the Model for End-Stage Liver Disease (MELD) score and the Child–Pugh score. Infection events, systemic inflammatory response syndrome (SIRS) and mortality rate were monitored for a 12-month period.

Patients with HIV, bacterial infection at hospital admission (culture-positive blood or AF and PMN > 250 cells/AF μl), on corticosteroid treatment, with immunodepression or any type of carcinoma or malignancy or ongoing antibiotic therapy were excluded from the study. The Hospital Ethics Committee approved the study protocol and informed consent was obtained from all patients enrolled in the study.

A cohort of 34 patients was included in our study and 64 samples of ascitic fluid and blood were collected. All these patients underwent one or more therapeutic paracenteses and, each time, the ascitic fluid was collected. For some patients, blood samples were collected at the same time. All the samples were analysed according to the standard microbiological method. Blood samples were inoculated in aerobic and anaerobic blood culture bottles maintained for four days at 37 °C. Ascitic fluids were inoculated in Columbia blood agar, mannitol salt agar, MacConkey agar and Sabouraud agar media. Moreover, an enrichment step was also performed for each AF sample by inoculating the fluid in blood culture bottles and incubating them in aerobiosis and anaerobiosis at 37 °C for four days. Only those clinical samples with a negative microbiological report were subsequently analysed.

Aliquots of ascitic fluid (15 ml) and serum, obtained after centrifugation of blood samples (5 ml), were centrifuged and the pellet suspended in 2 ml of sterile distilled water and stored at −80 °C.

About 800 μl of the sample was used to search for bacterial DNA (bactDNA), 200 μl for endotoxin test, 200 μl for peptidoglycan/β-D-glucan and 800 μl of the sample was used for observing the presence of damaged or intact bacterial cells under a microscope.

DNA extraction

DNA extraction was performed using a standard method. Briefly, an aliquot of 800 μl of sample was centrifuged at 10,000 × g for 10 min. The pellet was suspended in lysis buffer (Tris-EDTA buffer containing 10 % SDS) and the proteins were removed after digestion with proteinase K. Cellular debris was removed by phenol–chloroform treatment. Bacterial DNA was recovered by isopropanol precipitation. The extracted DNA was suspended in 100 μl of sterile nuclease-free water and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

PCR protocols

A nested polymerase chain reaction (PCR) protocol with universal primers BR2 and BR3 [17, 18], targeting a conserved bacterial chromosomal 16S rRNA gene region, was applied to the DNA extracted from each of the clinical samples: in the first step, a region of 1465 bp was amplified, while in the second step (nested PCR), an amplification region of 1137 bp was selected (Table 1). All the samples containing bacterial DNA and resulting positive to the nested PCR with universal primers were then subjected to two PCR protocols previously developed and targeting Enterobacteriaceae and

Table 1 Polymerase chain reaction (PCR) primers used in universal nested PCR and in PCR targeting Enterobacteriaceae DNA and *Enterococcus faecalis* DNA

Primer	Sequence	Amplified gene	Amplicon size (bp)	Primer specificity	Reference
BR2	Fwd: AGAGTTTGATCCTGGCTC Rev: GGTTACCTTGTTACGACTT	16S rRNA	1465	All bacteria	Weisburg et al. [17]
BR3	Fwd: CCTACGGGAGGCAGCAG Rev: GGTTACCTTGTTACGACTT	16S rRNA	1137	All bacteria	Muyzer et al. [18] This study
EntB	Fwd: TGGGGATGACGTCAAGTCAT Rev: TGAATCACAAAGTGGTAAGCG	16S rRNA	300 IS: 155*	Enterobacteriaceae	Thalheimer et al. [9] This study
pbp5	Fwd: CATGCGCAATTAATCGG Rev: CATAGCCTGTCGCAAAAC	pbp5	444 IS: 372*	*Enterococcus faecalis*	del Mar Lleó et al. (1999) [19]

*Amplicon size of fragment used as the internal standard (IS).

Enterococcus faecalis [9, 18]. The specific target for Enterobacteriaceae was in a common region of the 16S rRNA gene (*entB*), while that of *E. faecalis* was a 444-bp fragment located within the penicillin-binding protein 5-encoding gene (Table 1).

All PCR assays were carried out in a GeneAmp PCR System 9700 thermal cycler (Perkin Elmer). Each reaction was performed in a final volume of 50 μl. After an initial denaturation of 10 min at 95 °C, PCR was conducted with 30–35 cycles consisting of 40 s denaturation at 95 °C, 30–40 s annealing at adequate annealing temperature and a 40-s extension period at 72 °C, with a 15-min final extension at 72 °C. In each run, a negative control, containing sterile and nuclease-free water instead of DNA, and a positive control, consisting of purified *E. coli* or *E. faecalis* DNA, were included.

The PCR products were analysed in 2 % agarose gel electrophoresis stained by GelRed Nucleic Acid Gel Stain (Biotium, CA, USA) and were visualised by the ImageMaster Gel Analysis System (Amersham Pharmacia Biotech, Milan, Italy).

Amplification products were purified with the QIAEX II Gel Extraction Kit (Qiagen, Crawley, UK) and analysed by sequencing (BMR Genomics, Padova, Italy). The identification of DNA sequences was obtained by BLAST at the National Center for Biotechnology Information (NCBI) website.

Quantification of bacterial DNA by competitive PCR

A competitive PCR assay was applied to quantify the bactDNA present in the samples.

With regard to the samples containing *E. faecalis* DNA, competitive PCR using the internal standard pbp5 IS (Table 1), obtained as previously described [19], was applied for a quantitative evaluation of bacterial DNA. Similarly, for samples that were positive by EntB-PCR, an internal standard (IS) derived from the selected fragment of the *entB* region was prepared. Briefly, a fragment of 155 bp was eliminated from the *entB* sequence by cutting in two TfiI sites. The fragment obtained was amplifiable by the primers EntB Fwd and EntB IS (Table 1). Prior to PCR quantitative determination of specific DNA found in clinical samples, we constructed a standard curve using 10-fold dilution of *E. faecalis* and *E. coli* DNA suspensions in order to test the concentrations of DNA ranging from 100 ng to 100 pg.

PCR was conducted as described above, except that PCRs contained a constant amount of the appropriately diluted IS, as previously described [19, 20].

Detection of lipopolysaccharide and peptidoglycan/β-D-glucan in clinical samples

The E-TOXATE test (Sigma-Aldrich, Milan, Italy) and the Silkworm Larvae Plasma (SLP) Reagent Set (Wako Pure Chemical Industries, Osaka, Japan) were used, according to the manufacturers' instructions, to evaluate the presence of lipopolysaccharide (LPS) and peptidoglycan/β-D-glucan, respectively, in all samples from the patients included in the study. All samples were tested in duplicate.

Endotoxin detection procedure: an aliquot of 0.1 ml E-TOXATE Reagent Working Solution was added to each tube containing 0.1 ml of the clinical sample, E-TOXATE water (negative control) and endotoxin standard solution (positive control), and gently mixed. All samples were incubated for 1 h at 37 °C. After incubation, tubes were gently removed from the incubator and slowly inverted to observe the possible formation of compact gelatin. The presence of compact gel formation is considered a positive reaction. All other results (soft gels, turbidity and increase in viscosity or clear liquid) are considered a negative test, indicating the absence of LPS.

Peptidoglycan/β-D-glucan detection: for this assay, the SLP Reagent Set was used. This test is based on the natural activation of "prophenoloxidase cascade system" in the haemolymph of the Bombyx mori silkworm by the presence of peptidoglycan and/or β-D-glucan that causes the formation of melanin. 200 μl of each sample was added and mixed with

800 μl of sterile distilled water. Samples, sterile distilled water (negative control) and standard purified peptidoglycan preparation (positive control) were incubated for 10 min at 80 °C and, after ice-cooling, used for assay.

100 μl of each sample was mixed with 100 μl of SLP solution. The development of a black colour after incubation at 30 °C for 60 min is indicative of melanin formation and the test is considered positive.

Evaluation of the presence and integrity of bacterial cells

The presence of bacterial cells and their state of integrity was evaluated by treating the clinical samples with the Live/Dead kit (Live/Dead BacLight™, Molecular Probes, Eugene, OR, USA). The test was performed according to the manufacturer's instructions. This method is based on the use of a mixture of two fluorochromes, SYNTO9 and propidium iodide, with different capabilities of penetrating the bacterial wall and emitting fluorescence at two different wavelengths (green and red, respectively). 800 μl of samples were centrifuged at 14, 000 × g for 5 min and suspended in 50 μl of a 1 % NaCl solution. 5 μl of the dye mixture (3 μl SYNTO9 and 2 μl propidium iodide) was added to each sample. After 15 min of incubation in the dark, the sample was filtered (Amicon 0.2 μm) and the filter was directly positioned on a slide and observed by a fluorescence microscope (Leitz Wetzlar Orthoplan).

If the cell envelope is intact, the passage of only SYNTO9 that links to the DNA determines a green fluorescence staining (470–550 nm). If the cell membrane is partially damaged, both propidium iodide and SYNTO9 can enter the cell, with a resulting red fluorescence (630–650 nm) [21].

Statistical analysis

With regard to the possible association between microbiological data and clinical parameters in patients with liver disease, all the values are reported as mean ± standard deviation (SD) and p-values <0.05 were considered significant. Data were analysed as continuous or categorical by using the Student's t-test for parametric data and the Mann–Whitney U-test or the Wilcoxon test for non-parametric data. The Chi-square test was used for the comparison of dichotomist data. Logistic regression analysis was employed to identify possible predictors of infection and in-hospital mortality. Univariate analysis (log-rank) was used to identify prognostic factors of survival and the variables selected by this analysis were included in a multivariate analysis according to a Cox regression model. The survival curves were created using the Kaplan–Meier method. The software used for the analysis was Number Cruncher Statistical System (NCSS) 2007.

Results

Presence and quantification of bacterial DNA in culture-negative ascitic fluid and serum samples from cirrhotic patients with ascites

A total of 64 clinical samples, 46 of ascitic fluid and 18 of blood, were collected from 34 cirrhotic patients with bacterial culture-negative reports. All the samples were examined for the presence of bacterial DNA by applying the universal nested PCR, EntB-PCR and *E. faecalis* pbp5-PCR protocols.

As shown in Table 2, a total of 19/46 (41 %) AF samples and 4/18 (22 %) sera resulted positive to PCR targeting bacterial DNA. The bactDNA-positive samples were isolated from 16 different patients. As can be observed in Table 2, nine patients (AA, AB, AD, AH, AI, AJ, AK, AN and BE) had one AF sample positive for the presence of bactDNA. Another three patients (AE, AR and AX) presented two AF-positive samples. Moreover, four patients, AO, BB, BC and BG, showed the simultaneous presence of bacterial DNA in ascitic fluid and blood. All bactDNA-positive samples (23 samples) were subsequently subjected to EntB-PCR and PCR with pbp5 primers: 15 samples of AF and three sera were amplified by *entB* primers and three AF samples showed the presence of *E. faecalis* pbp5 (Table 2).

The amplified bacterial DNA isolated from clinical samples was associated to specific bacterial genus/species by sequencing. 74 % of samples (17/23 samples) contained DNA from Enterobacteriaceae. As indicated in Table 2, identification at the species level revealed that seven samples contained *E. coli* (patients AA, AE, AX and BC), five samples *Klebsiella* spp. (patients AH, AR and BG), three samples *Enterobacter* spp. (patients AN and BB), one sample *Serratia* spp. (patient AK) and one sample *Citrobacter freundii* (patient AI). The remaining 22 % of samples (5/23) corresponded to Gram-positive bacteria, namely *E. faecalis* (three samples, patients AB, AD and BE) and *Staphylococcus aureus* (two samples, patient AO, DNA amplicon identified by sequencing). It was not possible to identify the species corresponding to the DNA detected in the sample from patient AJ.

To evaluate the amount of bactDNA present in the culture-negative clinical samples containing Enterobacteriaceae DNA or *E. faecalis* DNA, protocols of competitive PCR using a specific IS were applied. The results obtained are reported in Table 3. The quantities of DNA varied largely among the different samples, with four samples (AH, AI, AR1 and AR2) resulting certainly significant (DNA concentrations ≥90 ng/ml) and specimens with low levels of DNA, such as samples AA and BE2 (DNA concentrations <10 ng/ml). Regarding the other positive samples, DNA concentrations yielded average values. The DNA extracted from the sample collected from patient AJ patient resulted not quantifiable.

Table 2 Presence of bacterial DNA in ascitic fluid samples and sera (s) determined by universal nested PCR (BR2, BR3), EntB-PCR and pbp5-PCR, and identification of bacteria (Bact ID) in our cohort of cirrhotic patients (PT)

PT	Nested PCR (BR2, BR3)	EntB-PCR	pbp5-PCR	Bact ID	PT	Nested PCR (BR2, BR3)	EntB- PCR	pbp5- PCR	Bact ID
AA	+	+	−	*E. coli*	AW 1s	−	−	−	
AB	+	−	+	*E. faecalis**	AW 2s	−	−	−	
AC	−	−	−		AX 1	+	+	−	*E. coli*
AD	+	−	+	*E .faecalis**	AX 2	+	+	−	*E. coli*
AE 1	+	+	−	*E. coli*	AX 2s	−	−	−	
AE 2	+	+	−	*E. coli*	AY	−	−	−	
AF	−	−	−		AYs	−	−	−	
AG	−	−	−		AZ	−	−	−	
AH	+	+	−	*Klebsiella* spp.	AZs	−	−	−	
AI	+	+	−	*C. freundii*	BA	−	−	−	
AJ	+	+	−	Undetermined	BB 1	−	−	−	
AK	+	+	−	*Serratia* spp.	BB 1s	−	−	−	
AKs	−	−	−		BB 2	+	+	−	*Enterobacter* spp.
AL	−	−	−		BB 2s	+	+	−	*Enterobacter* spp.
AM	−	−	−		BC	+	+	−	*E. coli*
AN	+	+	−	*Enterobacter* spp.	BCs	+	+	−	*E. coli*
ANs	−	−	−		BD	−	−	−	
AO	+	−	−	*S. aureus*	BDs	−	−	−	
AOs	+	−	−	*S. aureus*	BE 1	−	−	−	
AP	−	−	−		BE 2	+	−	+	*E. faecalis**
AQ	−	−	−		BE 3	−	−	−	
AR 1	+	+	−	*Klebsiella* spp.	BF 1	−	−	−	
AR 2	+	+	−	*Klebsiella* spp.	BF 2	−	−	−	
AS 1	−	−	−		BF 2s	−	−	−	
AS 1s	−	−	−		BF 3	−	−	−	
AS 2	−	−	−		BF 4	−	−	−	
AT	−	−	−		BF 5	−	−	−	
ATs	−	−	−		BG	+	+	−	*Klebsiella* spp.
AU	−	−	−		BGs	+	+	−	*Klebsiella* spp.
AUs	−	−	−		BH 1	−	−	−	
AV	−	−	−		BH 2	−	−	−	
AW 1	−	−	−		BH 2s	−	−	−	

Presence of other bacterial markers in AF and blood samples resulting positive for bacterial DNA

Table 3 shows, in addition to the presence of bactDNA and its quantification, the presence of lipopolysaccharide (LPS), peptidoglycan/β-glucan (PG/β-glucan) and bacterial cells (intact and damaged cells viewed under a microscope) in each one of the AF and blood samples examined. In regard to the blood samples, the microscopy evaluation with the Live/Dead kit was not applicable.

Endotoxin was detected in 14 AF and three blood samples containing bactDNA. As expected, LPS was detected in all samples containing Enterobacteriaceae DNA, with the only exception of the sample from patient AJ. Peptidoglycan/β-glucan was detected in some of the bactDNA-positive samples: the five samples containing Gram-positive DNA and another nine samples containing Enterobacteriaceae (Table 3). In 15 out of 19 samples of AF that registered positive for bactDNA, the presence of bacterial cells (intact or damaged) was microscopically appreciable. In three samples, collected from patients AA, AK and BE, even if fragments of bacterial wall were detected, intact and/or damaged cells were not observed. It should be noted that, in patient

Table 3 Quantity of bactDNA (DNA ng/ml) and presence of endotoxin (LPS), peptidoglycan/β-glucan (PG) and bacterial cells (Bact cells) in samples of AF and serum (s) collected from patients (PT). RC: fluorescent red cells, GC: fluorescent green cells, nd: not determined, nq: not quantifiable

Patient	DNA (ng/ml)	LPS	PG	Bact cells	Patient	DNA (ng/ml)	LPS	PG	Bact cells
AA	5	+	−	−	AW 1s	−	−	−	nd
AB	20	−	+	GC	AW 2s	−	−	−	nd
AC	−	−	−	−	AX 1	40	+	+	RC
AD	15	−	+	RC	AX 2	10	+	+	RC, GC
AE 1	20	+	−	GC	AX 2s	−	−	−	nd
AE 2	10	+	−	RC	AY	−	−	−	−
AF	−	−	−	−	AYs	−	−	−	nd
AG	−	−	−	−	AZ	−	−	−	−
AH	90	+	+	RC, GC	AZs	−	−	−	nd
AI	110	+	+	RC	BA	−	−	−	−
AJ	nq	−	−	−	BB 1	−	−	−	−
AK	10	+	−	−	BB 1s	−	−	−	nd
AKs	−	−	−	nd	BB 2	10	+	−	GC
AL	−	−	−	−	BB 2s	10	+	−	nd
AM	−	−	−	−	BC	10	+	+	RC, GC
AN	10	+	+	GC	BCs	15	+	+	nd
ANs	−	−	−	nd	BD	−	−	−	−
AO	nd	−	+	RC	BDs	−	−	−	nd
AOs	nd	−	+	nd	BE 1	−	−	−	−
AP	−	−	−	−	BE 2	5	−	+	−
AQ	−	−	−	−	BE 3	−	−	−	−
AR 1	150	+	+	RC, GC	BF 1	−	−	−	−
AR 2	120	+	+	RC, GC	BF 2	−	−	−	−
AS 1	−	−	−	−	BF 2s	−	−	−	nd
AS 1s	−	−	−	nd	BF 3	−	−	−	−
AS 2	−	−	−	−	BF 4	−	−	−	−
AT	−	−	−	−	BF 5	−	−	−	−
ATs	−	−	−	nd	BG	15	+	−	GC
AU	−	−	−	−	BGs	10	+	−	nd
AUs	−	−	−	nd	BH 1	−	−	−	−
AV	−	−	−	−	BH 2	−	−	−	−
AW 1	−	−	−	−	BH 2s	−	−	−	nd

AJ, all the bacterial markers, except bacterial DNA, resulted negative.

Based on the data presented in Table 3, an analysis of the concordance among the different bacterial markers was performed for all the clinical samples. All the samples that resulted negative to the presence of bactDNA (41 samples collected from 18 patients) also lacked the other bacterial indicators. Among patients for which samples contained bactDNA, nine (AB, AD, AH, AI, AN, AO, AR, AX and BC) showed a concordant presence of all the expected bacterial markers (endotoxin, PG/β-glucan and bacterial cells for Gram-negatives or PG/β-glucan and bacterial cells for Gram-positives). In five patients (AA, AE, AK, BB and BG), the bactDNA presence was not associated to detectable PG/β-glucan. Moreover, in patients AA and AK, the absence of detectable PG/β-glucan is coexistent with the lack of fluorescent bacterial cells.

Correlation between microbiological and clinical data in a series of cirrhotic patients with suspected bacterial infection

To perform a statistical analysis of the possible correlation between microbiological data and clinical parameters, patients were selected based on the following criteria: having samples with at least 10 ng of bactDNA and at least two of the expected bacterial markers. Twelve patients (AB, AD, AE, AH, AI, AN, AO, AR, AX, BB, BC and BG) complied with established criteria. These patients, together with another 18 patients whose samples lacked all the bacterial markers, were subjected to statistical evaluations. As reported in Table 4, no statistically

Table 4 Clinical parameters of the enrolled patients grouped on the basis of the presence/absence of bacterial markers in ascitic fluid and serum samples. Successive infections, systemic inflammatory response syndrome (SIRS) and death rate reported were registered during the 12-month follow-up of the patients

Clinical parameters	Patients negative for the presence of all bacterial markers (18/34), mean ± SD	Patients positive for the presence of at least three bacterial markers (12/34), mean ± SD
Age, years	55 ± 11	56 ± 8
Male sex, *n* (%)	13 (72.2)	8 (66.6)
MELD score >15, *n* (%)*	6 (33.3)	10 (83.3)
Mean Child–Pugh score	8.9 ± 0.3	9.35 ± 0.5
C-reactive protein (mg/L)*	11.7 ± 4.5	25.6 ± 7.4
Mean arterial pressure (mmHg)*	80.6 ± 9.5	69.25 ± 2.5
Heart rate (beats/min)	73 ± 18.5	74.5 ± 14.5
Blood WBC/mm^3	6485 ± 1064	5812 ± 3135
Serum creatinine (mg/dL)	1.67 ± 0.7	1.18 ± 0.8
Bilirubin (mg/dL)	4.3 ± 2.2	9.18 ± 5.7
Albumin (g/dL)	3.12 ± 0.25	3.1 ± 0.3
Prothrombin time (INR)	1.48 ± 0.38	1.98 ± 0.8
Platelets/mm^3 (150,000–400,000)	120,428 ± 30,300	113,214 ± 38,500
Haemoglobin (g/dL)	10.4 ± 2.95	10.36 ± 1.55
Serum sodium (mmol/L)	134.7 ± 8	128.57 ± 10
Successive infections, *n* (%)*	3 (16.6)	9 (75)
SIRS, *n* (%)*	1 (5.5)	7 (58)
Death rate (no. of days)*	220 ± 10	71 ± 8

*p-Value < 0.05

significant differences were observed in the baseline frequencies of age, sex, mean Child–Pugh score and in the greater part of basic haemodynamic characteristics between the two study groups. On the contrary, some clinical parameters showed a statistically significant correlation with the presence/absence of bacterial markers: for patients with positive bacterial marker samples, the C-reactive protein was significantly higher and the mean arterial pressure significantly lower when compared with patients without bacterial molecules ($p = 0.02$ and <0.001, respectively). A significant relationship exists also between the presence of bacterial markers and an MELD score value >15 ($p < 0.001$). Moreover, a significant correlation was also found between the emergence of subsequent infections (75 % of the positive patients vs. 17 % of patients with samples resulting negative for bacterial markers, $p < 0.05$) and SIRS (58 % of positive patients vs. 6 % of negative patients, $p < 0.05$) during a follow-up of one year. In regard to the survival curve of the different patients (Fig. 1), the mortality rate, in the 12-month follow-up of the patients, is significantly higher for the group with positive bacterial markers. Moreover, these patients have an average survival rate of 71 days compared with a survival rate of 220 days calculated for patients with bacterial markers-negative samples.

Discussion

Although the decision of treating or not a suspected case of ascites infection relies on PMN count, less than 40 % of these cases are culturable [22], and this fact, together with a frequent absence of clinical signs, may delay the diagnosis of bacterial infection in patients with severe liver diseases and the

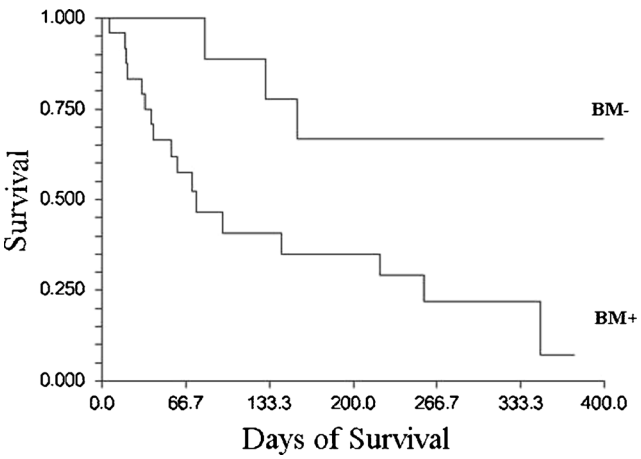

Fig. 1 Survival curves during a 12-month follow-up period in patients with cirrhosis according to the presence or absence of at least three considered bacterial markers (p-value = 0.01). *BM+*, patients registering positive for the presence of at least three bacterial markers (12/34); *BM–*, patients registering negative for bacterial markers (18/34)

application of a targeted antibiotic treatment. Our study started from the assumption that in vitro non-recoverable bacterial forms might be involved in suspected cases of infection in cirrhotic patients with culture-negative microbiological reports [23–25]. In many cases, culture-negative infections in cirrhotic patients derived from a bacterial translocation with the reaching of bacteria or bacterial molecules of ascitic fluid but not maintaining the recoverability, then resulting not culturable under standard conditions. Moreover, as a consequence of a former antibiotic treatment, viable but non-culturable bacteria or fragments of killed bacteria can persist in body sites, thus justifying the recovery of bacterial components. By using DNA amplification protocols, we detected the presence of bacterial DNA in 36 % of the culture-negative ascitic fluid and blood samples obtained from 16 of the 34 cirrhotic patients enrolled in the study. About 70 % of the involved non-recoverable bacteria were Gram-negative Enterobacteriaceae, a percentage similar to those reported by other authors [2, 6, 11], while the DNA detected in the other samples corresponded to *Enterococcus* and *Staphylococcus* species.

For 17 patients, it was possible to collect matched AF and peripheral blood samples. In this context, four patients showed the simultaneous presence of bactDNA in AF and serum, a fact that might be considered molecular evidence of bacterial translocation. In three cases (AK, AN and AX), the presence of bactDNA in AF was not associated with positivity of serum sample. Considering that the three AF samples contained a fairly low quantity of bactDNA, we believe, in agreement with other researchers [22, 26], that these cases may represent low bacterial colonisation of AF without systemic infection. Alternatively, it could be considered a residual permanence of bacterial cells in AF that, in the presence of stressed conditions, like low bactericidal capacity and the presence of immune inhibitory factors, were induced to activate bacterial survival mechanisms with a block of the division capability but maintenance of vitality [24].

Bacterial DNA is claimed to be a promising surrogate marker for BT, a frequent event in patients with cirrhosis and ascites without overt bacterial infections. Various reports indicated that it could be indicative of pre-clinical infection or a persistent source of inflammation in cirrhotic patients with ascites [2, 11, 22, 25]. However, the clinical significance of the presence of bacterial DNA in AF is not completely understood and has been questioned. Moreover, it is important to stress the need for an accurate interpretation of the results obtained with molecular methods because of their high sensitivity and the consequent risk of false-positive results.

To support the clinical significance of the presence of bactDNA in clinical samples with negative microbiological reports in evaluating the possibility of a bacterial infection, we analysed the concordance of the presence of DNA with other bacterial markers: the presence of microscopically visible bacterial cells, including damaged bacteria, of endotoxin, an important virulence factor of Gram-negative bacteria, and of components of the bacterial wall also having an immunogenic role. All the samples lacking bacterial DNA also resulted negative for the presence of the other bacterial markers. On the contrary, the samples that resulted positive for the presence of at least 10 ng of bactDNA also contained at least two of the expected bacterial markers, namely LPS, bacterial cells and, in a number of cases, components of bacterial cell walls. It has to be stressed that we encountered some difficulties in some cases in reading the results from the SLP Reagent Set assay (for the detection of peptidoglycan/β-glucan), which, in fact, resulted not sufficiently sensitive in the specific clinical samples examined. In one case (patient AJ), the only bacterial marker present was bacterial DNA, indicating a probable unspecific false-positive result or a high sensitivity of the test.

We believe that the detection of the other bacterial markers in bactDNA-positive clinical samples makes the bacterial presence and its clinical significance more realistic in that those bacterial molecules could be considered, taken as a whole, as early markers of a possible infection not yet fully developed. We then propose the detection of these markers as a complementary approach for diagnosing possible BT and subsequent infection in cirrhotic patients.

Our proposal of including a battery of bacterial markers in the monitoring of cirrhotic patients might also be helpful in establishing a clinical prognosis. A number of authors [2, 11, 13, 16] have reported that the bactDNA presence and other bacterial components in AF of patients with cirrhosis could be considered an indicator of poor diagnosis and worse prognosis. Also from our data, it is possible to deduce that patients with positive samples for at least three of the considered bacterial markers showed a worsening of liver functionality and an alteration of circulatory efficiency. The mortality rate of these patients was higher and death occurred in a shorter time. Moreover, only in those patients with samples containing the three bacterial markers were these more severe clinical conditions accompanied, during the follow-up, with a higher incidence of successive infections and SIRS.

In conclusion, the individuation in clinical samples of the various bacterial markers considered here may be an attractive approach for the early detection of bacteria, including also those that cannot be divided and recovered by standard microbiological methods based on bacteria culture. Early detection of bacteria components in

clinical samples might be useful for more selective and appropriate prophylaxis to prevent the development of serious bacterial infections and to avoid further and serious complications in cirrhotic patients with ascites.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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