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PCR-detected fungal infection is associated with fatal outcomes in cirrhotic patients with spontaneous peritonitis

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ABSTRACT

Background & aims: The rate of mortality from Spontaneous Peritonitis (SP) in cirrhotic patients is still high despite the development of new antibiotic treatments and intensive hospital care. The coexistence of spontaneous fungal peritonitis (SFP) is almost entirely ignored health problem, because it is difficult to be diagnosed at an early stage by conventional culture-based methods. Therefore, this study was designed to employ PCR-based method in evaluating the prevalence of fungal infection in cirrhotic patients with peritonitis who failed to respond to the recommended therapy and to determine its association with in-hospital mortality.
Subjects and Methods: A total of 80 cirrhotic patients admitted to the hospital with ascites (June-2013 to April-2015) were followed in this study.
Results: During hospitalization, 23 (42%) of patients had died although they received guideline-driven treatment. The demographics, clinical, hematological, and biochemical data were similar in both mortality and survival groups. However, the incidence of fungus infection was the only significantly elevated parameter in the mortality group than in the survival group (7/23;30% vs.0/32;0%, P = 0.0012). This fungal infection was significantly associated with SP drug resistance development (P = 0.007). Intriguingly, all the cases of fungal infection were detected by PCR-based method while culture-based diagnosis was able to detect the fungal infection in only 4 of these cases indicating a diagnostic sensitivity of 57%.
Conclusion: Our results reflect a strong association between SFP and in-hospital mortality in cirrhotic patients with SP that may offer a coherent explanation for the antibiotic treatment failure in such patients. Prompt PCR-detection and antifungal coverage is warranted in these cases.
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1. Introduction

Spontaneous Peritonitis (SP) is one of the most deadly life threatening complications of liver cirrhosis [1]. The key pathogenic mechanism initiating SP is the translocation of enteric pathogens, such as gram-negative bacteria, endotoxin, gram-positive bacteria, and fungi from the gut to peritoneal fluid. In cirrhosis, the rate of such pathogenic translocation increases due to the compromise of intestinal epithelial barrier integrity as well as immune surveillance mechanisms [2]. Early diagnosis and effective treatment of SP is essential to shorten hospital stays and reduce mortality. The diagnosis is established by ascitic fluid polymorphonuclear (PMN) count >250 cells/mm³ [3]. Additionally, ascitic fluid is routinely cultured in blood culture bottles to identify the pathogen. The most frequently isolated microorganisms are gram-negative bacilli [4]. Therefore, existing clinical guidelines of the International Ascites Club, the American Association for the Study of Liver Diseases, and the European Association for the Study of the Liver recommend third-generation cephalosporins as an empirical therapy for SP [5,6]. For acute SP, intravenous cefotaxime (2 gm/12 h for 5 days) is usually used, while prophylactic therapy entails norfloxacin (400 mg/day) [7]. Nevertheless, the rate of SP mortality is still high ranges from 40% to 70% despite the early diagnosis,

development of new antibiotic treatments, and intensive hospital care [8].

It is widely accepted that resistance to third-generation cephalosporins is the prevailing cause of mortality among all SP cases. This is evident from the high mortality rate among cirrhotic patients with infection by germs resistant to third-generation cephalosporins in comparison to those infected by pathogens that are sensitive to antimicrobials [9]. Third-generation cephalosporin-resistant SP is a common diagnosis when antibiotic treatment fails to drop the PMN count by at least 25% during 48 h [5]. Several mechanisms have been proposed regarding how antibiotic resistance occurs in SP. One such mechanism refers to the increased incidence of extended-spectrum-β-lactamase (ESBL) producing enterobacteriaceae, causing resistance to third-generation cephalosporins [10]. The other reported mechanism results in treatment failure with antibacterial agents in SP is the existence of fungal infection [11]. *Candida albicansis* is the most commonly detected fungus from ascitic fluid of cirrhotic patients and has been reported as potent etiological agents of SP. However, spontaneous fungal peritonitis (SFP) does not differ symptomatically from spontaneous bacterial peritonitis (SBP). Furthermore, SFP is difficult to be diagnosed at an early stage by conventional culture-based methods due to the longer time spent on fungal culture results and usually passed unnoticed. Because of these limitations, incidence of SFP was reported as rare with ill-defined clinical characteristics and prognosis [12].

These clinical difficulties impose the necessity of accurately detecting SFP at the earliest stage possible before the start of guideline-driven treatments. Recent diagnostic advances, including detection of fungal cell wall components such as galactomannan and B-glucan, or detection of fungal genomic DNA by polymerase chain reaction (PCR), would improve diagnostic ability and increase clinical awareness for SFP [13]. So far, studies evaluating the usefulness of these molecular techniques in the diagnosis of SFP in cirrhotic patients are scarce, especially from our region. Thus, the current study was undertaken to use DNA-based PCR method in evaluating the prevalence of fungal infection in cirrhotic patients with peritonitis who failed to respond to the recommended antibiotic therapy. Furthermore, the present work aimed to gain new insights into the clinical characteristics and prognosis of SFP in comparison with other groups of cirrhotic patients with non-fungal peritonitis.

2. Patients and methods

2.1. Population for analysis

The current investigation is a prospective case-control based study, which was approved by the ethical institutional review board at Mansoura University that complies with acceptable international standards. Written informed consent for participation was obtained from each participant. From June 2013 through April 2015, 80 case patients were recruited from the population of patients with diagnosed liver cirrhosis who were evaluated and treated at intensive care unit of hepatology and gastroenterology department in Mansoura University Hospital (MUH). The inclusion criteria were the presence of ascites with pathologically and ultrasonography confirmed liver cirrhosis and Egyptian residency. The severity of liver cirrhosis was assessed according to the Child–Pugh classification. The exclusion criteria were the presence of intra-abdominal surgery and secondary peritonitis. At the study entry, a thorough medical history was taken, physical examination was performed, and in-hospital mortality was assessed. The flowchart of the included cases is in Fig. 1.

2.2. Specimen collection and laboratory diagnosis

Blood and ascitic fluid samples were collected from each patient. The blood sample (5 mL) was used for complete blood picture and serum separation. Serum samples were assayed for liver function markers; albumin, bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Additionally, creatinine, random blood glucose, sodium and potassium levels were determined with commercial available kits.

Ascitic fluid (20 mL) was aspirated under all aseptic condition, before the initiation of antibiotic therapy. 10 mL of ascitic fluid was immediately inoculated at bedside into culture bottle while the other 10 mL was sent for biochemical analysis and PMN counting. For bacteria cultures, ascetic fluid samples were directly cultured and sub-cultured on blood as well as MacConkey agar plates at 37 °C. Identification of bacterial cultures was performed by gram staining and confirmed by coagulase test for gram positive cocci. For fungal cultures, ascetic fluid samples were directly cultured and sub cultured on Sabouraud's dextrose agar (SDA) for up to 14 days and examined by microscopy to detect fungal infection. In the biochemical analysis of ascitic fluid total protein content, albumin level, glucose level, and lactate dehydrogenase (LDH) activity were determined. The remaining ascitic fluid samples were used for DNA extraction followed by detection of universal fungal gene by PCR technique.

2.3. PCR detection of ascitic fungal DNA

Extracted DNA was detected with panfungal PCRs using ITS1 forward primer and ITS4 universal reverse primer, which amplify the ITS1 regions of fungal rDNA genes. The PCR mixture (25 μl) included the following: 12.5 μl of Taq PCR Master Mix, 1 μl of each primer, 2 μl of template DNA, and 8.5 μl nuclease-free water. The samples were then placed in a thermal cycler (MJ Research, Inc., USA) with amplification program as follow: One cycle at 95 °C for 5 min, followed by 30 cycles, each consisting of three steps; Denaturing step (30 s at 95 °C), annealing step (30 s at 55 °C), extension step (45 s at 72 °C), followed by one cycle at 72 °C for7 - min as a final step, A reagent blank, which contained all components of the reaction mixture with the exception of template DNA, was included in every PCR procedure. The PCR products were visualized on a UV transilluminator after electrophoresis on 2% agarose gels using ethidium bromide at 0.5 μg/ml.

2.4. Statistical analysis

The data collected in this study were processed and statistically analyzed by using IBM SPSS (Statistical Package for Social Science) software version 20 (Chicago, IL, U.S.A.). For descriptive statistics, frequency and percentage were calculated for qualitative variables, while the mean ± SD, median and range were used for quantitative variables. Qualitative data were assessed by Chi-Square test. Quantitative data were first checked for skewness and an unpaired *t*-test was performed if the distribution of the values was Gaussian. If the distribution was not normal, a non-parametric test, Mann-Whitney test, was used. P values less than 0.05 were considered to be statistically significant.

3. Results

A total of 80 patients were admitted with ascites due to cirrhosis during the study period, between 1 June 2013 and 30 April 2015. Of these, there were 55 (68.75%) patients with ascitic fluid infection (AFI) diagnosed by polymorph nuclear (PMN) count >250/mm^3. These patients were hospitalized, given the standard
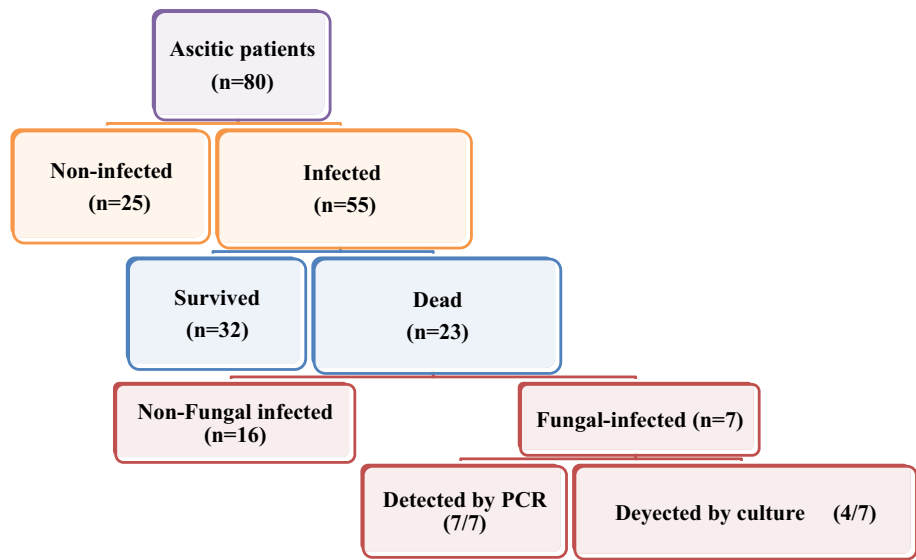
Fig. 1. The flow chart of the included cases. A total of 80 patients were admitted with ascites due to cirrhosis Of these, there were 55 patients with ascitic fluid infection (AFI) diagnosed by polymorph nuclear (PMN) count >250/mm^3. These patients were hospitalized and during hospitalization, 23 of these patients had died although they received guideline-driven treatment. The incidence of fungus infection was the only significantly elevated parameter in the mortality group than in the survival group (7/23; 30% vs. 0/32. All cases of fungal infection were detected by PCR-based method while culture-based diagnosis was able to detect the fungal infection in only4 of these cases.

Table 1
Demographic and clinical presentation of patients within survival and mortality groups.

Parameter	Survival group (n = 32)	Mortality group (n = 23)	P value
Age (years ± SD)	55.78 ± 9.3	58.74 ± 8.8	0.2401
Gender (M/F)	21/11	17/6	0.5668
Abdominal Pain (Y/N)	1/31	1/22	1.00
Jaundice (Y/N)	20/12	16/7	0.7745
Encephalopathy (Y/N)	9/23	7/16	1.00
Diabetes (Y/N)	7/25	4/19	0.7452
Child Paugh Score (b/c)	12/20	3/20	0.0660

Table 2
Hematological, biochemical, and liver function analysis of patients in survival and mortality groups.

Parameter (mean ± SD)	Survival group (n = 32)	Mortality group (n = 23)	P value
Hb (g/dL)	9.84 ± 1.82	9.72 ± 1.52	0.79
RBC counts (×10^6/mm^3 ± SD)	3.34 ± 0.89	3.33 ± 0.73	0.942
WBC counts (×10^3/mm^3 ± SD)	9.84 ± 1.82	9.72 ± 1.51	0.793
Platelet counts (×10^3/mm^3 ± SD)	155.81 ± 134.51	147.48 ± 115.97	0.81
Creatinine (mg/dL)	1.48 ± 0.99	1.88 ± 1.27	0.19
Blood glucose (mg/dL)	173.69 ± 132.09	165.13 ± 100.41	0.80
Sodium (mmol/L)	126.90 ± 8.16	128.04 ± 7.49	0.60
Potassium (mmol/L)	4.13 ± 0.72	4.11 ± 0.72	0.95
ALT (U/ml)	36.03 ± 22.36	36.78 ± 33.17	0.92
AST (U/ml)	70.55 ± 60.07	63.10 ± 55.52	0.64
Albumin (g/dL)	2.34 ± 0.42	2.36 ± 0.47	0.91
Bilirubin (mg/dL)	3.60 ± 2.95	3.70 ± 2.60	0.91
INR	1.55 ± 0.54	1.44 ± 0.28	0.43

Table 3
Biochemical analysis of ascitic fluid of patients in survival and mortality groups.

Parameter	Survival group (n = 32)	Mortality group (n = 23)	P value
SAAG (g/dL)	1.56 ± 0.46	1.48 ± 0.45	0.55
Albumin (g/dL)	0.79 ± 0.46	0.88 ± 0.41	0.46
Glucose (mg/dL)	92.83 ± 90.04	110.26 ± 107.53	0.52
Total protein (g/dL)	2.79 ± 1.12	2.69 ± 1.23	0.76
LDH (IU/L)	317.03 ± 420.42	266.05 ± 129.64	0.58
PCR-based Fungal detected infection (Y/N)	0/32	7/16	0.0012

treatment of third generation cephalosporin, and were included for analysis. During hospitalization, 23 (42%) of these patients had died although they received guideline-driven treatment. Therefore, further analysis was performed to find out factors associated with in-hospital mortality in AFI. The characteristics and laboratory data at admission of patients in the mortality and survival groups were compared. The demographics and clinical data of patients within both groups are shown in Table 1 and were matched for age, gender, clinical features, and complications such as abdominal pain, jaundice, encephalopathy diabetes, and Child-Paugh score. Hematological and biochemical laboratory tests including hemoglobin, red blood cell (RBC) count, white blood cell (WBC) count, platelet count, creatinine, blood glucose, sodium, and potassium levels together with liver function tests were similar in the two groups (Table 2). Furthermore, no differences in the serum ascites albumin gradient (SAAG) and the ascitic fluid analysis, including its concentrations of albumin, glucose, and total protein as well as lactate dehydrogenase activity, were found between the two groups. However, the highest incidence of fungus infection was the only significantly elevated parameter in the mortality group than in the survival group (7/23; 30% vs. 0/32; 0%, P = 0.0012) (Table 3). This reflects a strong association between fungal peritonitis and in-hospital mortality in cirrhotic patients with Spontaneous Peritonitis. Intriguingly, all the cases of fungal infection were detected by PCR-based method while culture-based diagnosis was able to detect the fungal infection in only 4 of these cases indicating a diagnostic sensitivity of 57%.

The aforementioned results provide a rationale to specifically analyze data from patients with spontaneous fungal peritonitis (SFP) in comparison with other groups with non-fungal AFI. The majority of included cases (41/55, 74.5%) had culture negative neutrocytic ascites (CNNA), characterized by PMN count >250/mm^3 and a negative ascitic fluid culture. However, there were 7 (12.72%) patients who had another variant of AFI, characterized

Table 4
Demographic and clinical presentations of patients with spontaneous fungal peritonitis (SFP) and those with culture negative neutrocytic ascites (CNNA).

Parameter	CNAA group (n = 41)	SFP group (n = 7)	P value
Age (years ± SD)	55.1 ± 9.48	59.85 ± 9.3	0.4893
Gender (M/F)	27/14	6/1	0.4087
Abdominal Pain (Y/N)	1/40	1/6	0.2730
Jaundice (Y/N)	26/15	4/3	1.0000
Encephalopathy (Y/N)	8/33	3/4	0.3271
Diabetes (Y/N)	8/33	1/6	1.0000
Child Paugh Score (b/c)	14/27	0/7	0.0662

Table 5
Hematological, biochemical, and liver function analysis of patients with spontaneous fungal peritonitis (SFP) and those with culture negative neutrocytic ascites (CNNA).

Parameter (mean ± SD)	CNAA group (n = 41)	SFP group (n = 7)	P value
Hb (g/dL)	9.81 ± 1.48	9.19 ± 1.84	0.32
RBC Counts ($\times 10^6$/mm^3 ± SD)	3.30 ± 0.72	3.28 ± 0.76	0.94
WBC Counts ($\times 10^3$/mm^3 ± SD)	8.11 ± 3.73	11.74 ± 6.14	0.04
Platelet Counts ($\times 10^3$/mm^3 ± SD)	146.24 ± 120.94	179.86 ± 158.21	0.52
Creatinine (mg/dL)	1.31 ± 0.84	2.43 ± 1.37	0.01
Blood Glucose (mg/dL)	163.24 ± 113.36	205.29 ± 127.18	0.38
Sodium (mmol/L)	128.02 ± 7.48	129.39 ± 5.03	0.65
Potassium (mmol/L)	4.06 ± 0.65	4.08 ± 0.82	0.68
ALT (U/ml)	38.33 ± 26.85	36.57 ± 38.36	0.88
AST (U/ml)	69.23 ± 55.52	66.57 ± 51.21	0.91
Albumin (g/dL)	2.37 ± 0.42	2.49 ± 0.49	0.50
Bilirubin (mg/dL)	5.40 ± 9.16	3.46 ± 3.27	0.58
INR	1.52 ± 0.17	1.30 ± 0.28	0.43

Table 6
Biochemical analysis of ascetic fluid of patients with spontaneous fungal peritonitis (SFP) and those with culture negative neutrocytic ascites (CNNA).

Parameter	CNAA group (n = 41)	SFP group (n = 7)	P value
SAAG (g/dL)	1.52 ± 0.46	1.59 ± 0.52	0.70
Albumin (g/dL)	0.85 ± 0.44	0.89 ± 0.49	0.80
Glucose (mg/dL)	100.29 ± 81.98	142.37 ± 178.97	0.31
Total protein (g/dL)	2.69 ± 1.22	2.98 ± 1.04	0.56
LDH (IU/L)	220.92 ± 208.99	374.29 ± 121.97	0.0026
Resistance to antibiotic (Y/N)	12/29	7/0	0.0007

by PMN count >250/mm^3 and a positive ascitic fluid culture without any evidence of external or intra-abdominal source of infection. The detected organisms were E. coli (57.14%), Staphylococcus aureus (28.5%), and Klebsiella (14.2%). Guided by these results, we proceeded to compare the biochemical and clinical outcomes between patients with SFP and patients with CNAA.

The demographics and clinical data of patients with SFP or CNNA are shown in Table 4. The two groups were matched for age, gender, clinical features, and complications such as abdominal pain, jaundice, encephalopathy and diabetes. According to the Child–Pugh classification, all SFP patients (100%) were class C while 27 patients in CNNA (65.9%) were class C. Majority of hematological and biochemical laboratory tests including hemoglobin, RBC, and platelet counts; blood glucose, sodium, and potassium levels together with liver function tests were similar in the two groups. Nevertheless, there were significantly higher level of creatinine and WBC counts in SFP group as compared to CNNA, p-value = 0.01 and 0.04, respectively (Table 5). Similarly, SFP group had a high LDH activity as compared to CNNA, P = 0.0026. There were no statistical differences in the SAAG and the ascitic fluid

analysis, including its concentrations of albumin, glucose, and total protein, were found between the two groups (Table 6).

Next we sought to delineate factors underlying the increased in-hospital mortality in patients with fungal peritonitis by investigating its association with the development of treatment failure. Treatment failure was defined as a <25% decrease in ascitic fluid PMN in follow-up paracentesis and persistence of signs of infection. As shown in Table 6, the fungal infection was significantly associated with SP drug resistance development (P = 0.007). Taken together, our results suggested that fungal peritonitis is a potential factor associated with substantially increased in-hospital mortality rate in cirrhotic patients with peritonitis due to the development of resistance to the standard regimen therapy that is characterized biochemically by increased creatinine level and increased LDH activity.

4. Discussion

Spontaneous Peritonitis (SP) is an important cause of morbidity and mortality in cirrhotic patients with ascites due to antibiotic treatment failure. There are multiple etiological factors affecting the response of SP to therapy, all of which vary by geographical location and have a direct impact on the characteristics of these patients, making SP an extremely complex condition [14]. The worldwide spread of extended-spectrum-β-lactamase (ESBL) producing enterobacteriaceae is the major culprit risk factor recognized for antibiotic treatment failure in SP [15]. Nonetheless, in 10–30% of patients with antibiotic-resistant SP, no defined cause is found. Therefore, it is conceivable that risk factors other than ESBL-producing bacteria exist for antibiotic treatment failure in SP, but the evidence is still unclear. The co-existence of fungal infection is usually an overlooked health problem that naively belittled in advanced medical care due to its difficulty to be diagnosed at an early stage by conventional culture-based methods. In this study, we put forward a new concept pertaining to the possible association of fungal infection with the development of treatment failure and subsequent mortality among cirrhotic patients with SP. Furthermore our study demonstrates a heretofore unreported utility of amplifying ITS1/rDNA by PCR as a sensitive and rapid method for diagnosing fungal infection in ascitic fluids from cirrhotic patients with SP.

Fungal infection and its toxin are well known in developing countries, such as Egypt, and have been implicated in a wide numbers of acute and chronic human diseases, including liver cancer and nephropathy [16,17]. However, the incidence of spontaneous fungal peritonitis (SFP) has been considered to be infrequent in cirrhotic patients and it is not well described [18]. This is due to the lack of clinical awareness for SFP, absence of the characteristic symptoms, and longer time spent on fungal culture to diagnose SFP [19]. It is therefore essential to use rapid and accurate non-culture-based methods for the detection of SFP. The ribosomal DNA (rDNA) is highly conserved in all fungal species, thus providing unquestionable information on the identification of fungal infection in body fluids and being an indicator of continuous exposure to such infection [20]. Accordingly, in the current work, the prevalence of fungal infection in our patients was evaluated with the help of this biomarker, which was amplified by PCR reaction using pan primer (IT1). The use of panfungal PCR in the detection of systemic fungal infection has been established previously and provides potential in terms of sensitivity and specificity. This input has originated partly from studies on whole-blood specimens from patients infected with fungi that showed panfungal PCR assay can detect multiple fungal genera [21]. These initial observations have been supported by showing the usefulness of Panfungal PCR for the diagnosis of fungal infection in neutropenic cancer patients [22],

haemato-oncology patients [23], immunocompromised patients [24], and in those with end-stage liver disease [19]. In concordance with these previous studies, our results demonstrate the superiority of Panfungal PCR over the conventional culture-based methods for the detection of fungal infection. However, our study is the first to report the potential utility of Panfungal PCR in detecting fungal infection in ascitic fluids from cirrhotic patients with SP. In our hospital, fungal peritonitis was detected in 12.3% of cirrhotic patients with SP with an incidence comparable to that of SBP characterized by PMN count >250/mm^3 and a positive ascitic fluid culture. Our results are in reasonable agreement with those of Hassan et al., who have shown the prevalence of SFP in Egyptian patients with end-stage liver disease to be 8.6%. This implies that fungi are not very rare causes of SP in our region which is almost 4 times higher compared to what has been reported in Republic of Korea (3.6%) [12]. This discrepancy may be explained not only by the difference in geographic location and in socio-demographic characteristics of the study groups, but also by the use of different method for detecting SFP.

Comparing the biochemical and clinical outcomes between SP cirrhotic patients with or without fungal infection, we found that patients with fungal infection had higher Child–Pugh score, increased creatinine level, and increased LDH activity and all died of the disease during hospitalization. Development of resistance to the standard regimen therapy may explain why SFP had a worse prognosis and a higher mortality compared with those with non fungal SP. Several mechanisms have been proposed regarding how fungal infection confers bacterial resistance to antibiotic therapy that can be applied to antibiotic-resistant SP seen in our cirrhotic patients. One such mechanism refers to the bacterial fungal interaction and the formation of a highly structured, often surface-associated, communities termed biofilms [11]. The formation of mixed-species biofilm creates a protected environment that promotes the resistance to both host clearance pathways and antimicrobial agents as well [25]. The extend of protection depends on the relative level of matrix polymers produced by both organisms within the biofilm. The more viscous matrix produced, the more effective it is in restricting the penetration of drugs [26]. Besides bacterial fungal biofilms, other reported effects might have contributed to the acquired bacterial antibiotic resistance in SP, such as interspecies communication [25], and changes in microbial behavior and survival that occur when bacteria and fungi occupy the same sites [11].

Collectively, our study identified fungal infection as a potential risk factor associated with increased in-hospital mortality in cirrhotic patients with SP pathogenesis. Our patients with fungal peritonitis were resistant to the standard regimen, had a worse prognosis characterized by higher Child–Pugh score, increased creatinine level, increased LDH activity, and higher mortality compared with those with non fungal SP. Considering these data, we envision the future clinical amalgamation of PCR-based fungal detection as well as antifungal coverage with existing guidelines for treating cirrhotic patients with antibiotic-resistant SP.

Conflict of interest

No conflicts of interest are declared by authors.

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