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Full Length Research Paper

Pathogens change in spontaneous bacterial peritonitis patients with cirrhosis

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The aim of this study was to determine the distribution of clinical microorganisms isolated from spontaneous bacterial peritonitis patients. Ascitic fluid was collected from these patients and cultured for pathogens. The patients were placed into three groups, Group A (01/1996 to 12/2000), B (01/2001 to 12/2005) and C (01/2006 to 06/2010) and the clinical data were compared among these groups. There was a significant difference in the ratio of pathogens (Gram- positive bacteria/ isolated pathogens, Gram-negative bacteria/ isolated pathogens) between groups A and C (P < 0.001). Spontaneous bacterial peritonitis patients with Gram-positive bacteria used significantly more antibiotics within 30 days compared to those with Gram-negative bacteria (P < 0.001) . In recent years, the types of isolated pathogens have significantly changed in Northern China. Such changes have also been observed in other countries and have been attributed to long-term antibiotic therapy and invasive procedures. Changes in the epidemiology of pathogens that cause spontaneous bacterial peritonitis must be monitored for optimal treatment.

Key words: Pathogens, gram-positive bacteria, spontaneous bacterial peritonitis, cirrhotic patients.

INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequent bacterial infection in patients with decompensated cirrhosis and ascites and it is associated with high mortality. Most organisms causing SBP are derived from the intestinal microbial flora (mainly enterobacteriaceae) and *Escherichia coli* is the most frequently isolated organism (Guarner et al., 1997) . In recent years, the etiology of SBP has undergone changes, and Gram-positive bacteria have emerged as the foremost cause of infection among patients. The pathogens of SBP in cirrhosis have changed during recent years (Campillo et al., 1998; Cholongitas et al., 2005; Park et al., 2003; Fernández et al., 2002; Singh et al., 2003).

Gram-positive bacteria were the predominant pathogens associated with SBP in a study by Singh et al. (2003). Although *Enterococcus faecalis* and *Viridans streptococci* were the most common Gram-positive pathogens, *Staphyllococcus aureus* accounted for 25% (5/20) of the Gram- positive bacteria (Singh et al., 2003). The emergence of Gram-positive bacteria, including *S. aureus*, as significant pathogens in SBP in recent years has been noted in other studies as well (Dupeyron et al., 2001; Campillo et al., 2002). Different authors observed the increasing incidence of SBP caused by Gram-positive bacteria in cirrhotic patients (Cholongitas et al., 2005; Campillo et al., 1998).

Angeloni et al. (2008) observed that an initial treatment with cefotaxime failed more frequently than expected. Their study supports that the microbial etiology of SBP has changed in recent years.

Most organisms causing SBP are derived from the intestinal microbial flora (mainly *enterobacteriaceae*) and *E. coli* is the most frequently isolated organism (Guarner et al., 1997). Because SBP is a serious complication in cirrhotic patients, empirical antibiotic therapy should be

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initiated before receiving the results of ascitic fluid cultures and should cover the most commonly isolated microbial organisms (Rimola et al., 2000). In recent years, primary or secondary quinolone prophylaxis in high-risk cirrhotic patients has been shown to decrease the incidence of SBP, as well as the mortality and the overall cost associated with SBP hospitalization (Aparicio et al., 1999). Unfortunately, there have been suggestions that the microbial etiology of SBP may have changed recently because of the increasing use of invasive procedures and quinolone prophylaxis (Fernández et al., 2002). In particular, long-term administration of norfloxacin prophylaxis in cirrhotic patients is associated with the isolation of quinolone-resistant Gram-negative bacteria from stool samples and the development of infections by these bacteria (Aparicio et al., 1999; Cereto et al., 2002). Moreover, norfloxacin prophylaxis and invasive procedures may promote carriage and bacterial infections because of multi-resistant Gram-positive bacteria (Fernández et al., 2002; Campillo et al., 2001). We therefore evaluated the characteristics and the possible changes of bacteria isolated from our cirrhotic patients with SBP during a recent 14-year period.

MATERIALS AND METHODS

Instrument

The bacteria were tested with Microscan WalkAway-40 automatic microbe analysis instrument produced by Date Behring Company, using Panel Type N1221 and NC31 (Date Behring company, USA) to test pathogens. The collection of ascitic fluid cultures use blood culture bottles (10 ml, BD Company, USA).

Origin of pathogens

All pathogens were isolated from ascites of SBP patients in our hospital during the period 1996 - 2010, the same bacteria were isolated two or more times from samples and the results were analyzed according to the National Committee for Clinical Laboratory Standards (NCCLS).

Patients

All 841 patients with decompensated cirrhosis, ascites and culture-positive SBP admitted to our Department between 1996 and 2010 were included in this retrospective study, but patients were excluded if tested positive to Human Immunodeficiency Virus (HIV) infection, heart failure or hepatocellular carcinoma. The diagnosis of cirrhosis was established on the basis of clinical examination, biochemical test and instrumental examination and/or liver biopsy. The severity of the liver disease in each patient was classified at entry according to the Child-Pugh scores (Pugh et al., 1973). The patients were placed in three groups, the 48 patients in group A were in hospital during the period of January 1996 to December 2000, the 48 patients in group B were in hospital during the period of January 2001 to December 2005 and the 84 patients in group C were in hospital during the period of January 2006 to June 2010. SBP was diagnosed by an ascitic fluid polymorphonuclear (PMN) cell count of > 250 cells/mm^3 and/or positive ascitic fluid cultures in the absence of clinical and laboratory evidence suggesting secondary peritonitis. Culture-positive SBP was defined as SBP with a positive ascitic fluid culture, meaning that a specific bacterium was isolated from culture bottles (Sheer and Runyon, 2005; Guarner and Soriano, 1997) . Medical records and laboratory data were reviewed. A medical history, physical examination, laboratory tests, diagnostic paracentesis and ascitic fluid cultures were performed according to our clinical practice in SBP patients.

Collection of ascites

Samples of 10 ml of ascitic fluid and blood were inoculated into aerobic and anaerobic blood culture bottles for bacteriological examination at the patient's bedside using a sterile technique and before the administration of antibiotic (Runyon et al., 1988). The samples were moved to the microbiology laboratory immediately. Antibiotic susceptibility was assessed using the disc diffusion method, with tablets of Neosensitabs (Rosco Diagnostica, Taastrup, Denmark), based on the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 1990). All organisms isolated in positive cultures were tested for antimicrobial susceptibility. A bacterium was considered resistant according to National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 1990).

Identification of pathogens

According to clinical microorganism operating instruction, all ascitic fluid cultures were inoculated into blood plate at 35°C and were cultured for 18 - 24 h, next the colonies were stained by Garm's stain and then picked single colony, finally bacterial confirmatory was carried out by American Dade Behring Company Microscan WalkAway-40 system.The strains were determined according to the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 1990).

Statistical analysis

Results were expressed as n (%) or mean values ± standard deviation. The statistical significance of the differences between the means of the experimental groups was tested by analysis of variance . The differences in proportion were tested by the chi-square test. A difference was considered statistically significant when $P < 0.05$. All data were analyzed using the statistical package SPSS software (version 12.0).

RESULTS

During the entire study period, SBP was diagnosed in 841 patients. Both blood culture and ascitic fluid culture positive patients were found (21.4%) of them. SBP was diagnosed in 180 patients and ascitic fluid cultures were found to be positive for all of them (there were 181 pathogens in the 180 patients; multiple organisms were isolated from 1 patient) . No significant differences were found between any two groups for gender, age or clinical situation. The clinical data were comparable between the groups. Gram-positive bacteria were significantly more frequently found to be the cause of SBP in Groups B and C than in Group A. Organisms isolated from the ascitic fluid of the patients with SBP throughout the study (1996–2010) and from Groups A, B and C were shown in

Table 1. Pathogens isolated from the ascitic fluid of cirrhotic patients from 1996-2000, 2001 - 2005 and 2006 - 2010.

Species	Period 1996-2000 (N=48)		Period 2001-2005 (N=49)		Period 2006-2010 (N=84)		Total (N=181)	
	n	%	n	%	n	%	n	%
Gram-negative bacteria								
Escherichia coli	22	45.8	11	22.4	18	21.4	51	28.2
Klebsiella ozytoca	9	18.7	3	6.1	6	7.1	18	9.9
Enterobacter cloacae	1	2.1	1	2.0	1	1.2	3	1.7
Flavobacterium breve	1	2.1	1	2.0	1	1.2	3	1.7
Xanthomonas maltophilia	0	0	1	2.0	2	2.4	3	1.7
Pseudomonad	0	0	1	2.0	1	1.2	2	1.1
Proteus	2	4.1	0	0	0	0	2	1.1
Gram-positive bacteria								
Staphylococcus aureus	2	4.1	8	16.3	15	17.9	25	13.8
Enterococci	4	8.3	0	0	0	0	4	2.2
Staphylococcus epidermidis	0	0	6	12.2	9	10.7	15	8.3
Group D streptococci	7	14.6	5	10.2	14	16.7	26	14.4
Streptococcus mitis	0	0	1	2.0	1	1.2	2	1.1
Streptococcus pneumoniae	0	0	1	2.0	1	1.2	2	1.1
Staphylococcus cohnii	0	0	1	2.0	6	7.1	7	3.9
Methicillin resistant Staphylococcus aureus (MRSA)	0	0	1	2.0	2	2.4	3	1.7
Staphylococcus heamolyticus	0	0	1	2.0	1	1.2	2	1.1
Staphylococcus warneri	0	0	1	2.0	1	1.2	2	1.1
Bacillus cereus	0	0	1	2.0	1	1.2	2	1.1
Fungi								
Cryptococcus neoformans	0	0	1	2.0	1	1.2	2	1.1
Mould	0	0	1	2.0	2	2.4	3	1.7
Torulopsis glabrata	0	0	1	2.0	1	1.2	2	1.1
Combined infection								
Group D streptococci	0	0	1	2.0	0	0	1	0.6
Enterococcus faecalis	0	0	1	2.0	0	0	1	0.6

Table 1. Clinical and laboratory data at the time of diagnosis were comparable for all groups. In Group A, all 48 patients with cirrhosis, 72.9% of the pathogens were Gram- negative bacteria. The predominant bacteria were *E. coli* (45.8%), *Klebsiella oxytoca* (18.7%) and Group D *streptococci* (14.6%). *E. coli* was the bacterium most frequently grown from ascitic fluid (Table 1). In Group B, in all 48 patients with cirrhosis, 53.1% of the pathogens were Gram-positive bacteria, among which *Staphylococcus* contributed the majority, accounting for 36.7% of cases, while *Streptococcus* accounted for 14.3% of the cases and *methicillin-resistant S. aureus (MRSA)* was isolated in 2.0% of cases (one isolate); 36.7% of the pathogens were Gram-negative bacteria among which *E. coli* accounted for 22.4% of the cases. *Eumycetes* accounted for 6.1% of all pathogens and combined infections accounted for 4.1%. Single organisms were isolated from 47 patients and multiple organisms were isolated from 1 patient (there were 49 strains in the 48 patients) (Table 1). Of the three strains of *Eumycetes*, there was one strain of *Cryptococcus neoformans*, one strain of *mycetes* and one strain of *Torulopsis glabrata*. Among two strains of combined infection, there was one strain of *Group D Streptococcus,* one strain of *E. faecalis*. Additionally, two positive cultures (a strain of *Corynebacteria* and a strain of *Micrococcus luteus*) were considered contaminated. These two cases were excluded from the analysis.

In Group C, in all 84 patients with cirrhosis, 60.7% of the isolated bacteria were Gram-positive bacteria and the most frequently found isolates were *E. coli*, *S. aureus* and *Group D Streptococcus*. This result was different from

Table 2. Baseline characteristics of patients with culture-positive spontaneous bacterial peritonitis during 1996 – 2000,2001-2005 and 2006 - 2010.

Patient characteristics	Period 1996 – 2000 (N = 48)	Period 2001 – 2005 (N = 49)	Period 2006 – 2010 (N = 84)
Mean age (years)	42.6 ± 5.4	43.1 ± 2.7	43.9 ± 3.7
Sex, males(%)	36(75)	39(80)	65(77.4)
Child-Pugh mean score, n (%)	11.3 ± 1.48	11.7 ± 1.88	10.7 ± 1.15
Cause of cirrhosis, n (%)			
HBV-related cirrhosis	32(75%)	35(71.4%)	59(70.7%)
HCV-related cirrhosis	7(14.6%)	8(16.3%)	13(15.5%)
Alcoholic cirrhosis	5(10.4%)	3(6.1%)	7(8.3%)
Other causes	4(8.3%)	3(6.1%)	5(5.9%)
Prior antibiotic treatment within 30 days, n (%)	25(52.1%)	33(67.3%)	59(70.2%)*
Gram-positive bacteria	13(27.1%)	26(53.1%)*	51(60.7%)*
Gram-negative bacteria	35(72.9%)	18(36.7%)*	29(34.5%)*
Fungi	0	6.1%	4.8%
In-hospital mortality rate, n (%)	8(16.7%)	9(18.4%)	15(17.9%)

Quantitative variables are expressed as mean values ± standard deviation or as n (%).*p < 0.05.

Table 3. Clinical characteristics of cirrhotic patients during 2001 – 2010 with culture-positive spontaneous bacterial peritonitis (SBP); arranged by the microbial agent

Patient characteristics	Gram-positive bacteria (N = 77)	Gram-negative bacteria (N =47)	P value
Mean age (years)	43.8±2.8	42.5±2.7	NS*
Sex, males (%)	22(84)	14 (77)	NS
Child-Pugh mean score	11 8 ± 1 8	11 3 ± 2 3	NS
Prior antibiotic treatment within 30 days, n(%)	70(90.9)	22(46.8)	0.05
SBP patients without symptoms	9(35)	6(33)	NS
In-hospital mortality rate, n(%)	7(26)	2(11)	NS

Quantitative variables are expressed as mean values ± standard deviation or as n (%). * NS: Not significant, P > 0.05.

many other reports. One positive culture (*M. luteus*) was considered contaminated and was excluded from the analysis.

Gram-positive bacteria were significantly more frequently found to be the cause of SBP in patients from Group C than in those from Group A. There was a significant difference in the constituent ratio (Gram-positive bacteria/ isolated pathogens, Gram-negative bacteria/ isolated pathogens) of pathogens between Group A and Group C (^{2} = 16.133, P < 0.001) . There was a significant difference in the constituent ratio (Gram-positive bacteria/ isolated pathogens, Gram-negative bacteria/ isolated pathogens) of pathogens between Groups A and B (^{2} = 9.630, P = 0.002). SBP patients in Group C used significantly more antibiotics within 30 days compared with those in Group A (^{2} = 4.351, P = 0.037) (Table 2).

Clinical characteristics of cirrhotic patients with SBP

In Groups B and C, no significant differences in the age, sex or child-pugh mean score were observed between patients with Gram-positive bacteria and those with Gram-negative bacteria. SBP patients with Gram -positive bacteria used significantly more antibiotics within 30 days compared with those with Gram-negative bacteria (^{2} = 29.646, P < 0.001) (Table 3) . Antibiotic susceptibility of multi drugs was observed in major pathogenic bacteria isolated from culture-positive SBP patients (Table 4).

DISCUSSION

SBP is a frequent complication of cirrhotic patients (Arroyo et al., 2000). Aerobic Gram-negative bacteria that

Table 4. Antibiotic susceptibility of major pathogenic bacteria during 1996 - 2000 and 2006 - 2010 (%).

Antibiotics	*Escherichia coli*		*Klebsiella ozytoca*		*Staphylococcus aureus*		*Staphylococcus epidermidis*	
	Period 1996- 2000	Period 2006 - 2010	Period 1996 - 2000	Period 2006 - 2010	Period 1996 - 2000	Period 2006 - 2010	Period 1996 - 2000	Period 2006 - 2010
Ciprofloxacin	51.2	42.5*	26.3	21.7	67.4	64.3	68.7	65.2
Amikacin	90.3	89.2	33.4	31.7	86.8	84.5	84.9	50.2*
Penbritin	46.5	19.3*	16.7	17.2	71.8	21.2*	72.6	17.1*
Cefazolin	48.6	33.4*	70.1	24.9*	69.9	67.8	70.5	65.3
Cefoxitin	95.7	94.1	86.7	78.3	98.7	97.3	97.8	96.6
Cefotaxime	66.6	37.4*	50.4	46.1	86.2	82.3	85.6	85.1
Ceftazidime	84.5	79.1	86.2	61.3*	86.3	81.5	86.7	82.4
Cefepime	86.7	51.1*	86.2	63.6*	86.5	83.4	88.1	86.3
Tienam	97.2	95.4	98.5	97.9	93.3	89.7	94.3	92.8

*$p < 0.05$.

translocate from the intestinal lumen are considered responsible for the majority of SBP cases (Guarner et al., 1995, 1997) . Nevertheless, in recent years, the etiologies for bacterial infections have undergone striking changes (Fernández et al., 2002).

Cholongitas et al. (2005) reported their results from Athens. Cases of culture-positive SBP in cirrhotic patients have more frequently been caused by Gram- positive bacteria in recent years (Cholongitas et al., 2005). Moreover, constituent ratio of pathogen is greatly diversified. This phenomenon is also confirmed by Campillo et al. (2002). They observed that Gram-positive pathogens were predominant among isolates from ascitic fluid cultures obtained from hospitalized cirrhotic patients with nosocomial SBP (Campillo et al., 2002). Jain et al. (1999) found that the most commonly isolated organisms were *coagulase- positive S. aureus* (44%), followed by *E. coli* (22%) (Jain et al., 1999). Our report also supported the view that Gram-positive pathogens were predominant among ascites fluid samples from SBP patients.

Why did the constituent ratio of pathogens change greatly over the past 14 years in China? We proposed three main possible reasons: First, in 2000, the guidelines for SBP in cirrhotic patients were published by the International Ascites Club. Third-generation cephalosporins, especially cefotaxime were one of the gold standard treatment (Rimola et al., 2000). Following a single episode of SBP, patients should have long-term antibiotic prophylaxis. Current treatments use third-generation cephalosporins or oral quinolones (Koulaouzidis et al., 2007).

Cefotaxime or other third-generation cephalosporins have been considered the first-choice empirical antibiotics in the treatment of cirrhotic patients with SBP (Strauss et al., 2006). At present, the third- generation cephalosporins are the most frequently used antibiotics for prophylaxis against SBP. Third-generation cephalosporins are the first-choice antibiotic because of a number of advantages (Koulaouzidis et al., 2009). They have a wider spectrum of action and lower toxicity than their predecessors and therefore are probably a better choice than other cephalosporins (Dongfang et al., 2008).

Antibacterial agents are widely prescribed in China for clinical use and account for more than 30% of all drug consumption in China (Xiao et al., 2008). The results presented here were from the Mohnarin study for the years 2004 – 2005 (Xiao et al., 2008).

In 1970, first-generation cephalosporins became available in China. First-generation cephalosporins were considered very effective in treating bacterial infections, especially against Gram-positive bacteria (Dongfang et al., 2008). However, these first-generation drugs were not effective against Gram-negative bacteria (Dongfang et al., 2008) . Consequently, second, third and fourth-generation cephalosporins, which were effective against Gram-negative bacteria, were generated.

Since 1985, third-generation cephalosporin use has increased; indeed, third-generation cephalosporins are considered to be more effective against Gram-negative bacteria than first-generation cephalosporins (Dongfang et al., 2008). Third-generation cephalosporins have been the first-line treatment of choice for SBP; another treatment option is oral quinolones (Koulaouzidis et al., 2007). Of the antibiotics used for SBP treatment, cefotaxime is the best-studied and has excellent penetration into ascites without nephrotoxicity (Sheer et al., 2005). Cefotaxime is considered the first-choice antibiotic for empirical treatment in cirrhotic patients with developing bacterial infections (Ricart et al., 2000). The incidence rate of SBP accompanied by Gram- negative bacteria continues to decrease yearly. The clinical efficacy of third-generation cephalosporins against *Staphylococci* is considered to be worse than first and second-generation (Dongfang et al., 2008). *Staphylococcus* contributes to the majority of SBP cases caused by Gram-positive bacteria. Indeed, the incidence

rate of SBP with Gram-positive bacteria has increased yearly.

In our study, we found that SBP patients with Gram-positive infections used significantly more antibiotics over a 30-day period than those patients with Gram-negative infections.

In short, the overuse of antibiotics over the past 20 years, especially the overuse of third-generation cephalosporins, may be causing the increasing frequency of Gram-positive bacteria in SBP cases in China.

Second, broad-spectrum quinolones are currently used for oral treatment of uncomplicated SBP (Strauss et al., 2006). Norfloxacin is widely used to prevent SBP in cirrhosis in China. Norfloxacin is also generally used in cirrhotic patients to reduce the risk of Gram-negative infections. Campillo et al. (1998) concluded that long-term administration of norfloxacin to cirrhotic patients reduced the risk of Gram-negative infections but increased the risk of severe hospital-acquired staphylococcal infections (Campillo et al., 1998).

The study performed by Llovet et al. (1997) showed the same results (Llovet et al., 1997). Our study confirms the validity of such an approach.

Quinolone prophylaxis has been shown to reduce the recurrence of SBP and to improve the survival of these high-risk patients (Bernard et al., 1999; Garcia-Tsao et al., 2001). However, there is a concern that the microbial causes of SBP may have changed in recent years with increasing involvement of quinolone-resistant Gram-negative and Gram-positive bacteria (Fernández et al., 2002; Llovet et al., 1997; Ortiz et al., 1999). Such changes have also been observed in neutropenic cancer patients (Carratala et al., 1996) and have been attributed to long-term antibiotic therapy as primary or secondary prophylaxis to high-risk cirrhotic patients (Ruynon, 2004).

Third, these epidemiological changes in the microbial causes of SBP have been associated with the increasing number of invasive procedures and hospitalization of cirrhotic patients in intensive care units, which promote the prevalence of Gram-positive bacterial infections and increase the incidence of infections caused by these microbial strains (mainly MRSA) (Campillo et al., 2001; Ortiz et al., 1999; Song et al., 2009).

Fernández found that infections caused by Gram-positive cocci had markedly increased in cirrhosis. This phenomenon may be related to the current high degree of instrumentation of cirrhotic patients (Fernández et al., 2002).

Another result showed that although the cases with a positive culture were few, the failure rate of cefotaxime therapy in the patients was very similar to that of the entire series (44%) (Angeloni et al., 2008). Song et al. (2009) proposed that ineffective initial therapies were responsible for the higher rate of treatment failure and mortality in SBP (Song et al., 2009). In these patients, because the isolated organisms were either intrinsically resistant to cefotaxime, capable of degrading expanded spectrum cephalosporins or inherently resistant to cefotaxime, cefotaxime failed. If this subgroup was small, Angeloni et al. (2008) thought their study supported that the microbial etiology of SBP was changing in recent years (Angeloni et al., 2008).

Although the use of antibiotics in the primary prophylaxis for SBP in patients with cirrhosis is controversial (Rohit et al., 2009), the extensive use of antibiotics in the management of cirrhotic patients still regularly occurs in China.

The clinical efficacy of third-generation cephalosporins for Gram-positive bacteria is worse than first and second-generation cephalosporins. SBP patients with Gram-positive bacteria had a higher mortality rate than those with Gram-negative bacteria in our study. Bernard Campillo observed that infections with *Staphylococci* were independently associated with a higher mortality rate (Campillo et al., 2002).

Third-generation cephalosporins failed to resolve the infection in 7 – 17% of patients with SBP (Felisart et al., 1985; Navasa et al., 1996). In more than 40% of SBP patients cefotaxime failed (Angeloni et al., 2008). Enterococcus infections is one cause of the failure of third-generation cephalosporin treatment of SBP because of their intrinsic resistance to cephalosporins (Moellering et al., 1984). The need to change this antibiotic treatment is higher than that reported in previous studies (Chen et al., 2005; Felisart et al., 1985; Navasa et al., 1996; Rimola et al., 1995; Runyon et al., 1991; Sheer et al., 2005).

Our observations confirm that the frequency of Gram-positive bacterial isolates from SBP patients has increased over the last 15 years. This phenomenon must be watched closely and taken into account when deciding on an appropriate treatment.

In conclusion, there has been an increase in the frequency of SBP caused by Gram-positive bacteria in recent years, but the precise reasons for this change need further investigation. The recent change in its pathogens may have some important implications for the treatment of SBP and they think a need for verifying the efficacy of current guidelines (Angeloni et al., 2008). The changes of bacteria isolated from our cirrhotic patients should be taken into account. Before our results (which are studied from an area) can be generalized, further investigation should be required.

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