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Original Article

Cost-effectiveness analysis on the surveillance for hepatocellular carcinoma in liver cirrhosis patients using contrast-enhanced ultrasonography

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Aim: Sonazoid is a new contrast agent for ultrasonography (US). Contrast-enhanced ultrasonography (CEUS) using Sonazoid enables Kupffer imaging, which improves the sensitivity of hepatocellular carcinoma (HCC) detection. However, there are no studies on the cost-effectiveness of HCC surveillance using Sonazoid.

Methods: We constructed a Markov model simulating the natural history of HCV-related liver cirrhosis (LC) patients, and compared three strategies (no surveillance, US surveillance and CEUS surveillance). The transition probability and cost data were obtained from published data. The simulation and analysis were performed using TreeAge pro 2009 software.

Results: When compared to the no surveillance group, the US and CEUS surveillance groups increased the life expectancy by 1.67 and 1.99 quality-adjusted life-years (QALY), respectively, and the incremental cost effectiveness ratio (ICER) were 17 296 $US/QALY and 18 384 $US/QALY, respectively. These results were both less than the commonly-accepted threshold of $US 50 000/QALY. Even if the CEUS surveillance group was compared with the US surveillance group, the ICER was $US 24 250 and thus cost-effective. Sensitivity analysis showed that the annual incidence of HCC and CEUS sensitivity were two critical parameters. However, when the annual incidence of HCC is more than 2% and/or the CEUS sensitivity is more than 80%, the ICER was also cost-effective.

Conclusions: Contrast-enhanced ultrasonography surveillance for HCC is a cost-effective strategy for LC patients and gains their longest additional life years, with similar degree of ICER in the US surveillance group. CEUS surveillance using Sonazoid is expected to be used not only in Japan, but also world-wide.

Key words: contrast-enhanced ultrasonography, cost-effective analysis, hepatocellular carcinoma, Sonazoid, surveillance

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fifth most common neoplasm in the world.[1] Although many environmental factors, including aflatoxins and alcohol,[2,3] have been implicated in the development of HCC, hepatitis B virus and hepatitis C virus (HCV) are the most important factors associated with the progression from chronic hepatitis to cirrhosis, and eventually to HCC.[4] Surveillance for HCC is recommended in patients with chronic liver injury to detect small-sized HCCs, which can be efficiently treated.[5] Ultrasonography (US) is a major surveillance method, because it provides low cost, real-time and non-invasive detection. However, there are some problems associated with this surveillance approach. It is known that the annual incidence of HCC increases with the degree of fibrosis.[6] Unfortunately, an increase in fibrosis makes US surveillance substantially more difficult, because the

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intrahepatic echo patterns in US become rough with advanced fibrosis.

Recently, a novel intravenous contrast medium for US, "Sonazoid", has become available in Japan. This strategy of using US with Sonazoid dramatically improves the sensitivity in the diagnosis of hepatic malignancy.[7] Thus, contrast-enhanced ultrasonography (CEUS) using Sonazoid can effectively detect HCCs that are usually overlooked by B-mode, which is currently used for observation. Therefore, this new contrast medium would be desirable for use in HCC surveillance. However, it is almost five times more expensive than the conventional observational approach in Japan. Until now, the surveillance for HCC using this novel agent has not been evaluated with regard to its cost-effectiveness, and this is the focus of the current study.

METHODS

WE USED TREE Age Pro 2009 (Tree Age Software Inc., William-stown, MA, USA) software to construct a Markov model, and estimated the cost-effectiveness of a surveillance program for HCC. The transition probabilities used in the analysis are listed in Table 1. The age specific mortality rate was obtained

Table 1 Values used in the analyses

Variable	Base value	Range	References
Excess annual mortality			
Child A Cirrhosis	0.02	0.00–0.08	8–11
Child B/C Cirrhosis	0.13	0.07–0.40	
Large HCC	0.90	0.50–1.00	12–14
Annual transition rate			
Child A to Child B/C	0.04	0.02–0.08	8,10,15,16
Small HCC to Large HCC (Undetected)*	0.30	0.10–0.60	17–19
Small HCC to large HCC (TAE treated)*	0.10	0.02–0.20	20–22
Annual incidence of HCC			
Incidence of new HCC	0.07	0.01–0.08	6,8,23–27
Incidence of HCC after curative treatment	0.20	0.10–0.37	13,25,28
Probability of small HCC at diagnosis	0.90	0.66–1.00	23,29
Test characteristics			
US			
Sensitivity	0.70	0.40–0.80	30–32
Specificity	0.90	0.70–0.90	
CEUS			
Sensitivity	0.90	0.80–0.95	7
Specificity	0.95	0.80–0.95	
Cost data			20,23,31,33–37
US	61		
CEUS	248		
Confirmation test	862	170–1 100	
LC	587	300–1 200	38
Decompensated LC	6 422	6 422–23 000	38
Terminal care	5 556	5 000–42 000	38
Resection	19 390	12 000–40 000	39
RFA	10 333	35 000–11 000	39
TAE	7 778	35 000–12 000	
Health-related QOL			40
Child A	0.75	0.66–0.83	
Child B/C	0.66	0.46–0.86	
HCC	0.64	0.44–0.86	

*Per 6 months. The costs were $US/6 months, and the baseline cost has been adjusted to US dollars (Currency rate: $1.00 = \90.00).
CEUS, contrast-enhanced ultrasonography; HCC, hepatocellular carcinoma; LC, liver cirrhosis; QOL, quality of life; RFA, radio-frequency ablation; TAE, transcatheter arterial embolization; US, ultrasonography.

from the homepage of the Japanese Ministry of Health, Labour, and Welfare.

Decision model

We estimated the long-term outcomes of different treatments by modifying a previously published computer simulation model[41] using current data on the natural history of chronic hepatitis C in Japan (Fig. 1). Each cycle consisted of 6 months. During each cycle, patients died according to the population-based mortality.

The decision tree for our analysis was composed of three arms: (i) the no surveillance group or "no surveillance" (ii) the B-mode US surveillance group or "US group", and (iii) the CEUS surveillance group or "CEUS group".

Assumptions 1 (program)

Based on the limited information available in the literature, the following assumptions were made:

1 the transition data from liver cirrhosis (LC) to decompensated LC are constant regardless of the patient's age and prior history of HCC;
2 the progression from compensated to decompensated cirrhosis is irreversible;
3 the incidence of HCC is the same in compensated versus decompensated cirrhosis.
4 the probabilities of HCC recurrence and growth remain constant over time;
5 surgery is not performed in patients with a background of decompensated cirrhosis or HCC recurrence; and
6 liver transplantation is not the first-choice for HCC therapy because it is still very rare in Japan.

Assumptions 2 (surveillance)

With regard to surveillance, the following assumptions were made:

1 HCC can be divided into two categories: "small" and "large". Small tumors (1–5 cm in diameter, and no

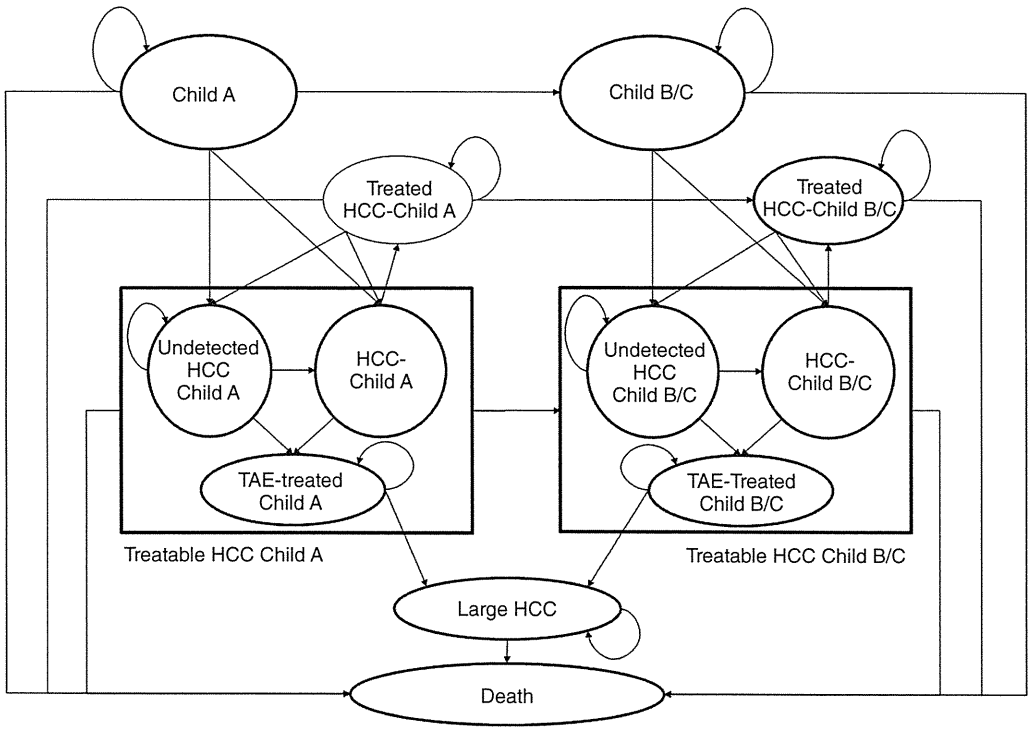

Figure 1 Natural history model. The arrows represent possible transitions during each 6 month cycle. Patients enter this model with Child A cirrhosis, and might develop Child B/C cirrhosis, hepatocellular carcinoma (HCC), both Child B/C and HCC, or death. If the health status does not change, then the patients remain in the same state of health. Surveillance and treatment strategies were superimposed on this model. TAE, transcatheter arterial embolization.

more than three in number) are asymptomatic, and remain undetected until the surveillance is performed. Large tumors are symptomatic, and the patient can receive palliative treatment only;
2 there are no small HCCs that can be detected incidentally in the no surveillance group;
3 patients with positive surveillance tests undergo a confirmatory test. [CT and either MRI (70%) or liver biopsy (30%)];
4 the test performance is independent of previous test results;
5 compliance with the program is 100%; and
6 there is a small rate of false-positive diagnoses, which will be discovered before any treatment.

The tumor growth rate was calculated with the assumption of a doubling time of 120 days.[17,18,42]

Since one year's worth is different in the health status, health-states utility should be taken into account for cost effectiveness analysis. Thus, we obtained the health-state utility information from meta-analysis.[40] The survival and costs were also discounted at the commonly accepted annual rate of 3%, because time and cost of distant future are generally thought to be of less value than those of present time.

Cost

The cost data shown in Table 1 are from data published in Japan, because Sonazoid is currently available only in Japan.

The data were converted to US currency at the exchange rate of US$1.00 = JP¥90.00. The cost of transcatheter arterial embolization (TAE) was estimated by including health insurance reimbursement using the reimbursement data in our hospital, because there were no available national data.

Sensitivity analysis

The results obtained from this model depended on the values that were used in the study; therefore a one-way sensitivity analysis was performed on all variables.

RESULTS

Accuracy of our model

TO VALIDATE THE model's accuracy, we compared this model's survival rate with the cumulative survival rates of 417 compensated cirrhosis patients obtained from a large European cohort clinical study under surveillance.[43] When we set the annual incidence rate of HCC as 4% to fit the European model, these two survival curves were very similar, and the accuracy of our model was validated (Fig. 2).

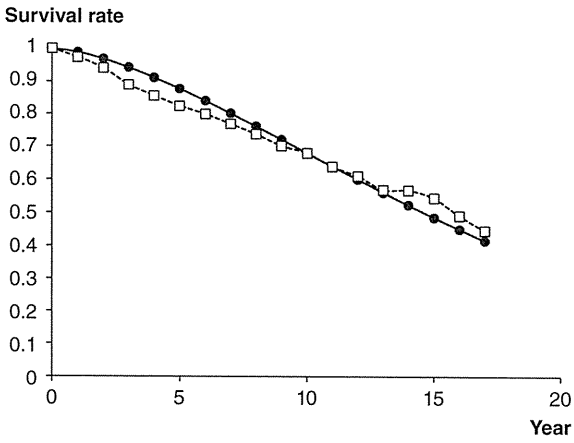

Figure 2 Comparison of the survival curves for compensated cirrhosis states between the one predicted by the current model and published data from a large cohort study.[43] Both data sources yielded similar curves. –●–, our model; -□-, Sangiovanni *et al.* 2004[43].

Baseline analysis

The expected life years of each group according to the starting age of the surveillance are shown in Figure 3.

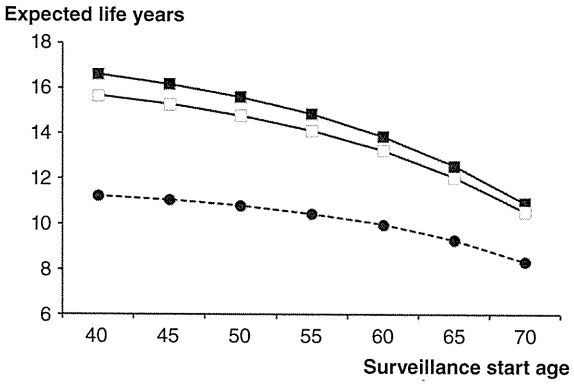

Figure 3 Expected life years according to surveillance at a starting age before it was discounted, and adjusted by health-state utilities. Although the expected life years decreased with age, both the ultrasonography (US) and contrast-enhanced ultrasonography (CEUS) surveillance groups increased the life expectancy even in 70-year-old patients. CEUS surveillance achieved the greatest gain in life expectancy in all analyzed age groups. –■–, CEUS Surveillance; –□–, US Surveillance; -●-, No Surveillance.

Table 2 Baseline analysis

Strategy	Total cost (US$)	Incremental cost (US$)	Expected life years (year)	QALY	Incremental QALY	IECR (US$/QALY)
No surveillance	29 142	–	10.45	6.18	–	–
US surveillance	58 064	28 922	14.13	7.85	1.67	17 296†
CEUS surveillance	65 726	36 584	14.86	8.17	1.99	18 384† (24 250‡)

†Compared with the no surveillance group.
‡Compared with the US surveillance group.
CEUS, contrast-enhanced ultrasonography; ICER, incremental cost effective rate; QALY, quality-adjusted life-year.

Both the US group and the CEUS group could extend their additional life years as compared with the no US group, regardless of age. The CEUS group could also extend their additional life years as compared with the US group. The biggest difference in expected life years between the US and CEUS groups was 0.93 at an age of 40 years. The superiority of surveillance with CEUS over US was also seen in the 70 year-old patients group. If the sensitivity of US was lower than 50%, then CEUS could extend their additional life years by 2 years and more as compared with the US group.

In the no surveillance group, 55 year-old patients (base value) with compensated HCV-related cirrhosis are expected to live 10.45 life years. When surveillance for HCC with conventional US or CEUS was used in these patients, their expected life years increased by 3.68 years and 4.41 years, respectively. Since the discount rate and health-related utility should be considered in cost-effective analysis, we showed the results of the baseline cost-effectiveness analysis in Table 2. Even though the additional expected life years became small when the program was analyzed while considering the discount rate and health-related utility, in comparison to having no surveillance, the US and CEUS groups still showed an increase in QALYs, 1.67 and 1.99 QALYs, respectively.

Next, the incremental cost-effectiveness ratio (ICER) was estimated, which is a measure of the extra cost incurred to save one year of life. The ICER of the US and CEUS groups, as compared to the no surveillance group, were $US 17 296/QALY and $US 18 384/QALY, respectively. These values were well below $US 50 000/QALY, which is commonly considered to be the cost-effective threshold. Even when the CEUS group was compared with the US group, the ICER of the CEUS group was $US 24 250/QALY, and was also cost-effective.

Sensitivity analysis

The above results depended largely on the baseline values used in this model, but the estimates of these parameters vary in the published literature. We therefore examined the effects of changing the value of each parameter through sensitivity analysis (Fig. 4). After performing the sensitivity analysis on all parameters in this model, three important parameters emerged in CEUS surveillance compared with US surveillance: the annual HCC incidence rates, and the CEUS sensitivity, and the US sensitivity.

Figure 5a shows the differences of ICERs in varying the US sensitivity. The ICERs of the US and CEUS groups were also less than US$ 20 000, and cost-effective when compared with the no surveillance group. On the other hand, when the CEUS group was compared with the US group, the ICER of the CEUS group increased as the US sensitivity increased up to almost the CEUS sensitivity. However, if the US sensitivity was 80%, then the ICER was $US 34 143, and still less than the threshold of $US 50 000/QALY. If the US sensitivity was lower than 60%, then the ICER of the CEUS group was almost $US 20 000, and thus was more cost-effective. On the other hand, CEUS sensitivity was especially affected when the CEUS group was compared with the US group, and the ICER rose sharply when the CEUS sensitivity was lower than 80% (Fig. 5b).

DISCUSSION

IN THE PRESENT study, we analyzed the cost-effectiveness of CEUS for HCC surveillance using Sonazoid in liver cirrhosis patients, and demonstrated that CEUS surveillance could cost effectively extend the expected life years, even compared with the US surveillance.

Currently, there are only two US contrast agents, Sonazoid and Levovist, which can be used for Kupffer imaging in the post-vascular phase. However, Levovist bubbles are very fragile, and are collapsed by US emissions easily. Therefore, Kupffer imaging in the post-vascular phase using Levovist needs to be performed by a single sweep scan of the liver, which is insufficient for surveillance.

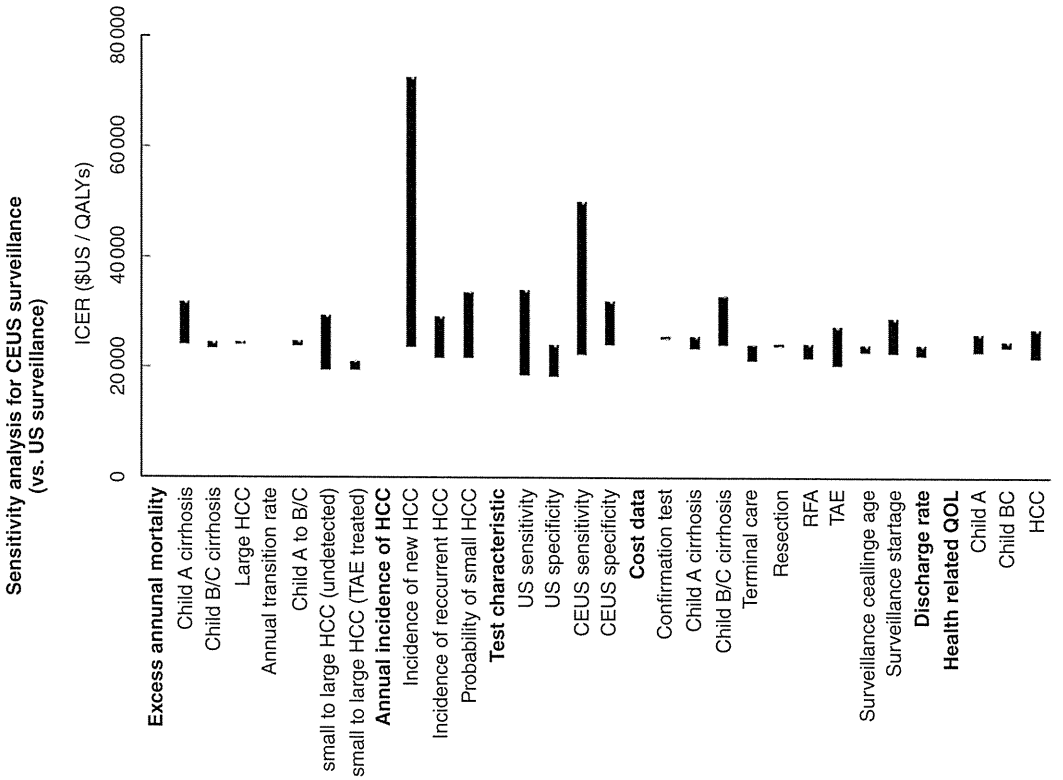

Figure 4 One way sensitivity analysis of the incremental cost-effectiveness ratio (ICER) for the contrast-enhanced ultrasonography (CEUS) surveillance group. When the ICER of the CEUS group was compared with the ultrasonography (US) group, the annual incidence rate of hepatocellular carcinoma (HCC) and CEUS sensitivity were critical parameters in this model. QALY, quality adjusted life year; RFA, radio-frequency ablation; TAE, transcatheter arterial embolization.

Sonazoid is composed of a hard shell containing bubbles, and produces stable, non-linear oscillations in the low-power acoustic field. Because of this feature, Sonazoid provides detailed perfusion features during vascular imaging in the vascular phase, and Kupffer imaging in the post-vascular phase at least 10 min after injection, without collapsing the bubbles. Specifically, Sonazoid CEUS is stable for at least 3 h after injection and allows for multiple and real time scans, because the Sonazoid microbubbles are phagocytosed by Kupffer cells.[44] In contrast, malignant hepatic tumors including HCC contain few or no Kupffer cells, which lead to clear negative contrast as a perfusion defect in Kupffer imaging.[45,46] Thus, surveillance for HCC using Sonazoid is especially useful for LC patients whose liver parenchyma have become roughened by fibrosis. For these reasons, the trend towards the use of US contrast agents in Japan has changed dramatically from Levovist to Sonazoid after it became commercially-available in 2007.

A recent study on the cost-effectiveness of surveillance for HCC reported the sensitivity of US at only 28.6% for detecting middle-sized HCC (between 2 and 5 cm in diameter).[47] The sensitivity of US depends on the skill of the operator, especially in LC patients, in which the intrahepatic echo patterns become roughened with advanced fibrosis. In sensitivity analysis, the US sensitivity was an important factor. When the US sensitivity is expected to be low due to patient physiologic factors such as obesity, CEUS surveillance is recommended. US technicians whose skill may not achieve the average level are also advised to perform additional CEUS using Sonazoid.

Contrast-enhanced ultrasonography sensitivity was a critical factor for cost-effectiveness. When the CEUS group was compared with the US group, and CEUS surveillance was not cost-effective if CEUS sensitivity was lower than 75% (Fig. 5b). As noted earlier, CEUS using Sonazoid is effective for Kupffer imaging, and it

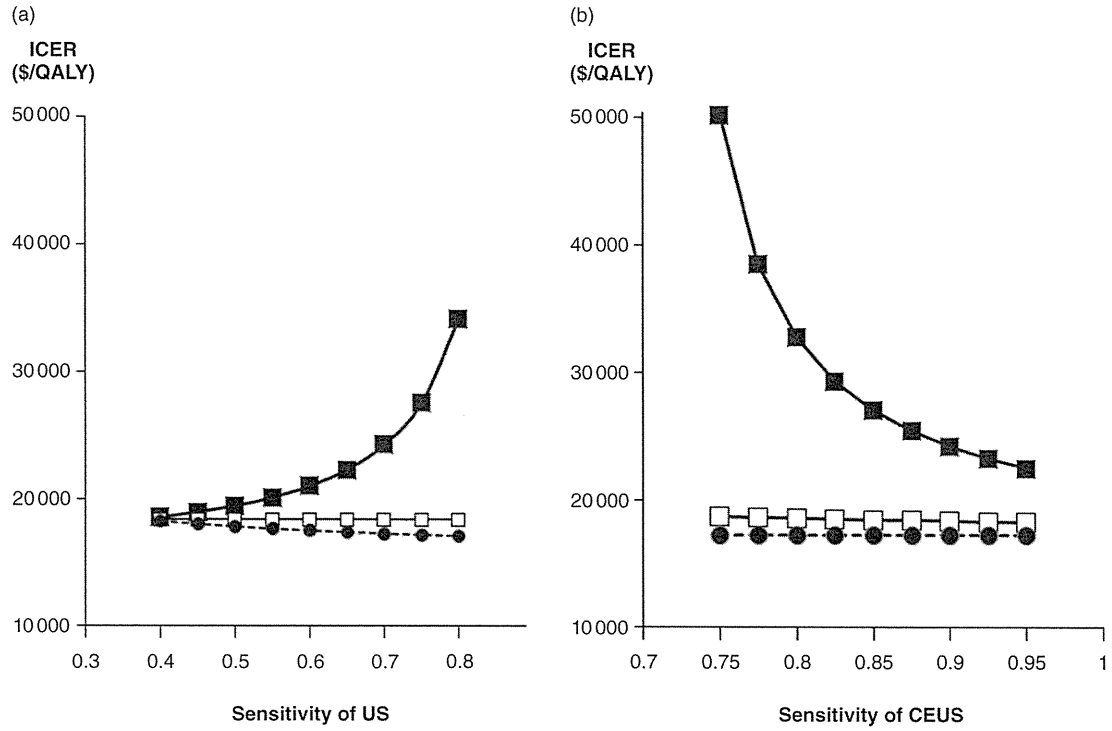

Figure 5 Effects of the sensitivity of ultrasonography (US) (A) and contrast-enhanced ultrasonography (CEUS) (B). When the incremental cost-effectiveness ratio (ICER) was compared with the no surveillance group, both US and CEUS surveillance groups were less than $US 20 000/quality adjusted life year (QALY) in all ranges. The ICER of the CEUS surveillance group achieved $US 50 000/QALY when the sensitivity of CEUS was lower than 0.75. -■-, CEUS (vs US surveillance); -□-, CEUS (vs no surveillance); -●-, US (vs no surveillance).

has been reported to have high sensitivity.[7] This helps technicians to detect the HCC more easily. Thus, a greater than 75% sensitivity represents a reasonable value for Sonazoid CEUS.

The incidence of HCC is the most critical parameter in decision-making for the surveillance of patients with cirrhosis. In our baseline analysis, we selected 7% as a baseline value because most studies in Japan reported 5–8% as the incidence of HCC.[6,26,48,49] This rate is slightly higher than the one in the United States and Europe, where incidence rates are reported from 1.5 to 4%.[8,27] Figure 6 shows how the incidence rate affects the ICER. When the ICER of the CEUS group was compared with the US group, it increased as the rate decreased. However, when the rate was 2%, the ICER of the CEUS group was still less than $50 000/QALY.

Although our results enable us to evaluate the effectiveness of CEUS surveillance, the study has some limitations. First, Sonazoid is available only in Japan. Thus, there are only Japanese published reports for analysis. On the other hand, our baseline data of US sensitivity 70% could be affected by the regional difference, and might be estimated lower than in the Japanese one. However, even if the US sensitivity was as high as 80%, ICER was still lower than $US 40 000 when CEUS surveillance was compared with US surveillance (Fig. 5a).

Similarly, our results were analyzed based on some hypothesis. Thus, the validation is desirable but is difficult because there are also ethical issues. For the solution of the problems, we performed the sensitivity analysis with the widest possible range using many representative reports. As the results of our analysis, we could indicate that the parameters except the HCC incidence rate, US sensitivity and CEUS sensitivity have little impact on cost-effectiveness.

In summary, our analysis suggests that surveillance for HCC in patients with compensated HCV-related cirrhosis by CEUS using Sonazoid was a cost-effective strategy. Since this cost-effectiveness decreased when the HCC incidence rate was low, this strategy should be selected

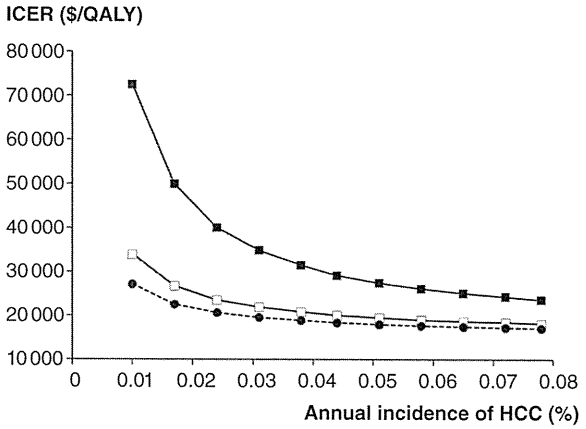

Figure 6 Effects of the annual incidence of hepatocellular carcinoma (HCC). The incremental cost-effectiveness ratio (ICER) values of the ultrasonography (US) and contrast-enhanced ultrasonography (CEUS) surveillance groups were less than $US 35 000/ quality adjusted life year (QALY) in all ranges as compared with the no surveillance group. However, the ICER of the CEUS surveillance group achieved $US 50 000/QALY as compared with the US surveillance group when the incidence was lower than 0.016. -■-, CEUS (vs no surveillance); -□-, CEUS (vs US surveillance); -●-, US (no surveillance).

considering of the influence of patient factors such as age, gender and fibrosis grade.

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Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Cirrhosis

Development of a new *in situ* hybridization method for the detection of global bacterial DNA to provide early evidence of a bacterial infection in spontaneous bacterial peritonitis

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Background & Aims: Despite the importance of identifying the causative pathogen(s), ascitic fluid cultures are occasionally negative in patients with spontaneous bacterial peritonitis (SBP). A novel strategy using the *in situ* hybridization (ISH) method was introduced to detect the bacterial genomic DNA phagocytized in the blood of patients with sepsis. In the present study, we developed a new ISH probe to detect global bacterial DNA (named as GB probe) and evaluated its utility for detecting the phagocytized bacterial DNA in SBP ascites.

Methods: Hybridization of bacterial DNA with the GB probe was examined by dot-blot and ISH tests. In addition, the utility of the ISH method to detect the bacterial DNA in the leukocytes of SBP ascites was evaluated.

Results: The GB probe hybridized with the genomic DNA of all 59 bacterial strains tested (59 species of 36 genus). Eleven of 51 patients with ascites (out of total 542 cirrhotic inpatients) were categorized as SBP. The ISH tests showed positive results in 10 of 11 SBP cases. However, the ISH tests all showed negative results in the 40 non-SBP ascitic samples. Therefore, the ISH tests yielded highly sensitive and specific results for detecting the phagocytized bacterial DNA in the leukocytes of SBP ascites. Moreover, all of the ISH test results were obtained within only one day.

Conclusions: Our newly established ISH method was found to provide both a rapid and sensitive detection of bacterial DNA in SBP ascites, thus suggesting its utility for providing early and direct evidence of bacterial infection in SBP ascites.

Keywords: Liver cirrhosis; Ascites; Bacterial infection; Phagocytization; Genomic DNA.

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Abbreviations: SBP, spontaneous bacterial peritonitis; ISH, *in situ* hybridization; PMN, polymorphonuclear neutropils; PCR, polymerase chain reaction; rRNA, Ribosomal RNA.

Introduction

Spontaneous bacterial peritonitis (SBP) is a severe life-threatening complication in cirrhotic patients with ascites, and its prevalence in hospitalized patients ranges between 10% and 30% [1–3]. Although the identification of the pathogen(s) is important for the management of infectious diseases, it takes several days to detect the casual bacteria from ascitic fluid cultures. Furthermore, despite the use of sensitive methods, ascitic fluid cultures are negative in about 10–60% of patients with the clinical manifestations of SBP [4–7]. On the other hand, the elevated count of polymorphonuclear neutrophils (PMN) in the ascitic fluid has been proven to be a very useful and sensitive method for diagnosis of SBP. A PMN count in ascitic fluid equal to or over 250/μl is definitive for SBP [8]. Since early diagnosis and treatment play a key role in the treatment of SBP, antibiotic therapy must be started immediately after the diagnosis of SBP, without knowledge of the causative organisms and their *in vitro* susceptibility. Therefore, SBP is an infectious disease that is usually diagnosed only by the PMN count in the ascitic fluid without the identification of the pathogen. Although it is clinically very important to confirm the bacterial infection, a definitive method for early detection of the bacterial pathogen in ascitic fluid has not been well established.

In order to identify the causal bacteria in sepsis, we previously reported a novel strategy using an *in situ* hybridization (ISH) method to detect the genomic DNA of bacteria phagocytized in neutrophils and macrophages [9–11]. Our previous studies established the utility of the ISH method by demonstrating evidence supporting the presence of a bacterial infection. The ISH method was almost four times more sensitive than the blood culture (42% *vs.* 11%) in detecting the causal bacteria of sepsis [10]. Furthermore, the results of ISH tests can be obtained within 1 day, while several days to 2 weeks are required for the results of the culture method. The aim of the study was to assess the utility of the ISH method by representing the evidence of bacterial infection in SBP ascites. An original probe was generated to detect the genomic DNA of broad-spectrum bacteria, and its utility to demonstrate the causative bacteria in SBP ascites was evaluated. The ISH test succeeded in achieving a rapid and sensitive detection of

intra-cellular bacteria in SBP ascites, thus suggesting this new method to be an effective tool to obtain direct evidence of a bacterial infection in SBP patients.

Patients and methods

The purpose and design of the study

SBP is one of the potentially fatal complications of cirrhotic patients with an infection of ascitic fluid. However, ascitic fluid cultures are often negative in patients with the clinical manifestations of SBP. In the present study, we developed a new ISH probe to detect global bacterial DNA and assessed its utility for detecting the phagocytized bacterial DNA in the leukocytes of SBP ascites.

The present study was an observational, cohort, and prospective study. The cohort/group included the consecutive cirrhotic patients who admitted to our department from January 2007 to March 2011. Ascitic fluid was obtained by paracentesis according to the usual clinical management of such patients. The condition evaluated in the study was SBP, and the intervention was ascitic liquid puncture. The purpose of the study was to evaluate the efficacy of the ISH test to offer direct evidence of a bacterial infection in SBP patients.

Study population

All cirrhotic patients with ascites admitted to our department from January 2007 to March 2011, were included in the present study. Cirrhosis was diagnosed by histological criteria and/or by clinical (laboratory, endoscopic and/or ultrasonographic) findings [12]. Exclusion criteria included the presence of any intra-abdominal, surgically treatable source of infection. Cases possibly affected by the antibiotic treatment, were also excluded from the analysis. Most of the 542 cirrhotic patients were admitted to receive the invasive treatment for hepatocellular carcinoma or esophageal varices, and their ascites was well controlled before admission. Only 58 cirrhotic patients could provide sufficient ascitic samples, and four patients were excluded due to receiving antibiotic treatment (one patient was treated due to the suggestive clinical manifestations of SBP in another hospital before admission to our hospital). In addition, three patients (one patient with hepatocellular carcinoma and two patients with pancreatic cancer) were excluded due to the complication of peritonitis carcinomatosa. Finally, 51 consecutive cirrhotic patients who met the inclusion criteria were analyzed in the present study (Fig. 1). All the patients had evidence of advanced liver disease, and Child–Pugh classification was grade B in 15 patients and grade C in 36 patients. Cirrhosis was diagnosed as non-viral hepatitis in 28 patients and chronic viral hepatitis in 23 patients (HBV 6, HCV 16, both 1). Non-viral cirrhotic patients were associated with alcoholic, autoimmune, and cryptogenic cirrhosis. The characteristics of the study population are summarized in Table 1. The study protocol confirmed the ethical guidelines of the 1975 Helsinki declaration, and patients were enrolled after providing their written informed consent to participate in this study. Blood samples were obtained to perform routine studies, including hematological, biochemical, and coagulation tests.

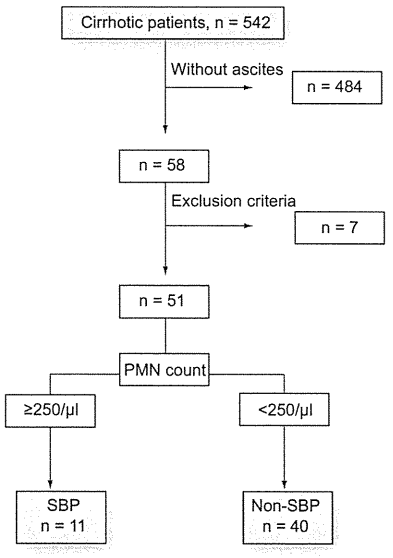

Fig. 1. Algorithm for the classification of cirrhotic patients.

Table 1. Characteristics of the study population.

Age (years)	62.5 ± 1.2
Gender (Male/Female)	35/16
Etiology (HBV/HCV/Both/Non-viral)	6/16/1/28
Child-Pugh classification (A/B/C)	0/15/36
Child-Pugh score	10.24 ± 0.20
Total bilirubin (mg/dl)	2.3 (0.4-28.3)
Albumin (g/dl)	2.68 ± 0.05
Prothrombin time (%)	61.2 (24.0-96.2)
Creatinine (mg/dl)	1.45 ± 0.15
MELD score	17 (7-40)
Hepatocellular carcinoma (present/absent)	13/38
Variceal hemorrhage (present/absent)	4/47
History of SBP or sepsis (present/absent)	3/48
Use of β-blocker (present/absent)	4/47
Use of PPI (present/absent)	40/11

HBV, hepatitis B virus; HCV, hepatitis C virus; PPI, proton-pump inhibitor. Data are expressed as the mean ± standard deviation or median (range).

Paracentesis

All 51 cirrhotic patients underwent diagnostic paracentesis to search for SBP in aseptic conditions following the usual procedures, and a routine biochemical study was carried out and the PMN count of the ascitic fluid was determined. The diagnosis of SBP was based on the elevated PMN count (⩾250/μl) in the ascitic fluid. Distinction from secondary bacterial peritonitis was performed according to the criteria previously reported [3,4,13,14]. Ascitic fluid culturing was carried out using both aerobic and anaerobic blood-culture bottles.

Probes

A series of grouped probes (designated SA, SE, PA, EF, and EK) were previously established for the specific detection of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and groups of enterobacteria (*Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae*), respectively [9]. These probes were generated to detect the agents that most frequently cause bacteremia and specifically find the intended pathogen.

The present study attempted to develop a new probe which could detect all kinds of bacteria. A cDNA probe for the 23S ribosomal RNA (rRNA) gene was thought to serve this purpose, because 23S rRNA is common to all bacteria. Therefore, polymerase chain reaction (PCR) was performed as described previously [15], and several cDNA fragments that corresponded to the 23S rRNA genes of various bacteria were obtained. Although every generated cDNA probe for 23S rRNA gene could detect the genomic DNA of plural bacteria, no probe could cover all kinds of bacteria with sufficient sensitivity (data not shown). This suggested that each type of bacteria had own unique sequences in their 23S rRNA genes, and DNA sequences were partially different from one another. Therefore, it would be difficult to establish a single cDNA probe that could solely detect all kinds of bacteria universally. Finally, plural cDNA fragments were mixed and used as new probe cocktails. This strategy succeeded in detecting the genomic DNA of all bacteria examined. The new probe mixtures were designated as the "global bacteria (GB) probe". The GB probe consisted of cDNA fragments for 23S Ribosomal RNA genes of various bacteria, including *S. aureus*, *Bacteroides fragilis*,

Table 2. Probes used for the *in situ* hybridization.

Probe	Detective pathogen
SA	*Staphylococcus aureus*
SE	*Staphylococcus epidermidis*
PA	*Pseudomonas aeruginosa*
EF	*Enterococcus faecalis*
EK	*Escherichia coli* *Enterobacter cloacae* *Klebsiella pneumoniae*
GB	Global Bacteria

Pseudomonas aeruginosa, *Enterococcus faecalis*, and *E. coli* (Genbank Accession No.: X68425, CR626927, Y00432, AJ295306, EU146962, respectively). Therefore, the probe cocktails called "GB probe" have been developed to detect the genomic DNA of whole bacteria, but the probe mixture cannot specifically identify the kind of bacteria. In contrast, the previously established probes can detect only limited types of bacteria, but can specifically identify the targeted pathogens (Table 2).

Dot-blot hybridization of the GB probe

Genomic DNA was isolated from various bacterial strains, including both Gram-positive and Gram-negative bacteria, and dot-blot hybridization was performed as described previously [9] to examine the utility of the newly developed GB probe for the detection of global bacterial DNA. Genomic DNA samples extracted from the fungi were used as negative control samples.

In order to determine the lower limit of detection of bacterial DNA, samples containing serially diluted genomic DNA of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100,000, 10,000, 1000, 100, 10, and 1 pg/μl). In addition, samples containing serially diluted DNA fragments that corresponded to the 23S rRNA genes of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strain were also prepared (final concentration; 100, 10, 1, 0.1, 0.01, and 0.001 pg/μl). Two microliters of each sample was spotted on the membrane, and these were used for the detection of bacterial DNA with the GB probe.

In situ hybridization of the GB probe in bacterial smear samples

In order to examine whether the ISH test could detect and visualize the genomic DNA of a single bacterial cell, smear samples of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared. In addition, a representative smear sample of a fungus (*C. albicans*: NBRC1594) was also prepared as a negative control sample. The slides were fixed for 20 min in Carnoy's solution, and ISH was performed according to the methods described previously [9–11].

In situ hybridization of the GB probe in an experimental infection model

A previous study [16] successfully detected intracellular bacteria in mouse phagocytic cells by the ISH method. The present study used human leukocytes extracted from blood samples derived from a member of our laboratory. First, various bacterial strains were phagocytized by leukocytes *in vitro*, according to the methods described in the published patent (US 7651837 B2: "Method for detecting and identifying organism causative of infection"), and the ability of the GB probe to detect intracellular bacteria was investigated. In addition, leukocytes which ingested fungus were also prepared and used as negative control samples. The procedures used for the ISH test were performed as previously described [9–11], and positive signals were detected in the cellular cytoplasm as purple-brown stains.

Next, in order to determine whether the ISH test can detect the phagocytized bacterial DNA in fluid samples with a lower concentration of leukocytes, we prepared samples with various leukocyte concentrations of approximately 1000, 300, and 100/μl. The samples containing these dilutions of leukocytes were spiked with either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacteria. After the 1-h spiking procedure, leukocytes were collected and the ability of the GB probe to detect the bacterial DNA was investigated.

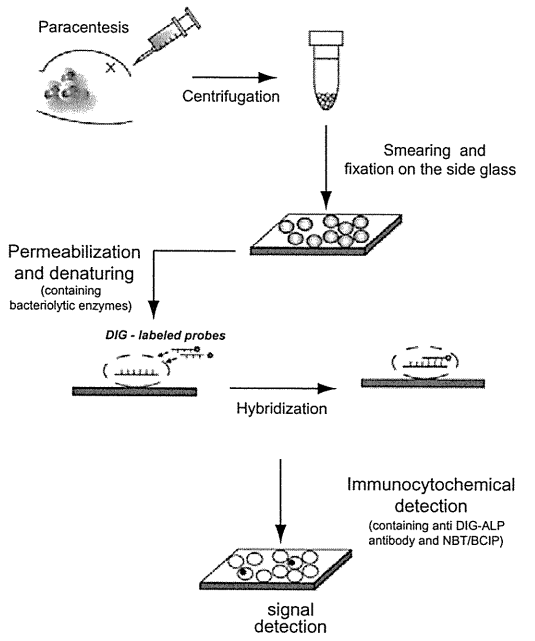

Fig. 2. Schematic representation of the *in situ* hybridization (ISH) for ascitic leukocytes. Floating leukocytes in ascitic fluid were collected by centrifugation and samples were spread on glass slides and prepared for ISH. ISH was performed according to the methods described in "Patients and methods".

Detection of the bacterial DNA in ascitic leukocytes

Ascitic samples were obtained by routine paracentesis as described above. Leukocytes were collected from ascitic fluid in the research laboratory, and ISH tests were performed to detect the bacterial genomic DNA. The procedure of ISH was based on the previously reported method with some modifications. In brief, migrated leukocytes in the ascitic fluid were collected by centrifugation (140–180*g* for 10 min). Bloody ascites underwent hemolysis with hypotonic buffer, and the leukocytes were re-collected by centrifugation and re-suspended in PBS (concentration: 5×10^4/μl). A 5–10 μl sample of the cellular suspension was spread onto a glass slide, and then allowed to air-dry. The slides were fixed for 20 min in Carnoy's solution, and the ISH was performed according to the methods as previously described [9–11]. A schematic illustration of the method is shown in Fig. 2. The ISH tests with all probes (GB, SA, SE, PA, EF, and EK probes) were performed simultaneously.

Statistical analysis

Baseline differences between SBP patients and non-SBP patients were statistically evaluated. Quantitative variables were expressed as the mean values ± SD, and were evaluated using Student's *t* test. Data with an abnormal distribution were expressed as the median values (range) and were evaluated using the Mann–Whitney *U*-test.

Results

Characteristics of patients and clinical data

Fifty-one cirrhotic patients with ascites (out of 542 cirrhotic inpatients) were consecutively included in the present study, based on the inclusion and exclusion criteria as described in

Table 3. Clinical data of the groups distributed according to the absence or presence of SBP.

	SBP (n = 11)	Non-SBP (n = 40)	*p value
Age (years)	63.5 ± 4.3	62.2 ± 1.0	0.63
Gender (Male/Female)	7/4	28/12	0.54
Etiology (HBV/HCV/Both/Non-viral)	0/5/0/6	6/11/1/22	
(Viral/Non-viral)	5/6	18/22	0.77
Child-Pugh classification (A/B/C)	0/3/8	0/12/28	0.78
Child-Pugh score	10.64 ± 0.51	10.13 ± 0.22	0.30
Total bilirubin (mg/dl)	2.4 (0.9-18.5)	2.2 (0.4-28.3)	0.32
Albumin (g/dl)	2.66 ± 0.11	2.69 ± 0.06	0.82
Prothrombin time (%)	52.7 (24.0-71.1)	63.0 (35.0-96.2)	0.09
Creatinine (mg/dl)	1.58 ± 0.37	1.42 ± 0.17	0.68
MELD score	19 (10-40)	16.5 (7-34)	0.12
HCC (present/absent)	2/9	11/29	0.53
Variceal hemorrhage (present/absent)	3/8	1/39	0.0068*
History of SBP or sepsis (present/absent)	2/9	1/39	0.050
Use of β-blocker (present/absent)	2/9	2/38	0.15
Use of PPI (present/absent)	9/2	31/9	0.76
PMN count of ascites (cells/μl)	1767 (281-20,593)	9.5 (0-168)	6.3 x 10^{-6}*

HBV, hepatitis B virus; HCV, hepatitis C virus; PMN, polymorphonuclear neutrophils; PPI, proton-pump inhibitor.

*A significant *p* value was a *p* value <0.05. Data are expressed as the mean ± standard deviation or median (range).

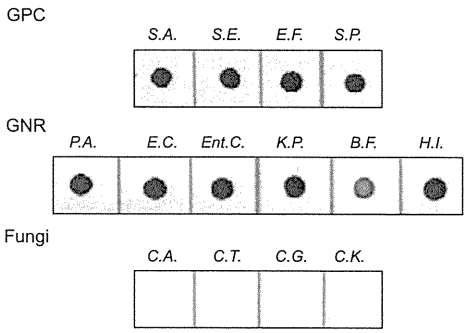

Fig. 3. Dot-blot hybridization of the GB (designed for Global Bacteria) probe with genomic DNA of various bacterial strains. Genomic DNA was isolated from various Gram-positive and Gram-negative bacterial strains, and dot-blot hybridization was performed as described previously [9]. Genomic DNA samples extracted from several fungi were used as negative control samples. Positive signals were observed in the DNA spots of various Gram-positive (GPC) and Gram-negative (GNR) bacterial strains. In contrast, the GB probe did react with the fungal DNA spots (Fungi). The names of bacterial and fungal strains shown are: S.A., *S. aureus* ATCC12600; S.E., *Staphylococcus epidermidis* ATCC14990; E.F., *Enterococcus faecalis* JCM5803; S.P., *Streptococcus pneumoniae* ATCC39938; P.A., *Pseudomonas aeruginosa JCM5962*; E.C., *Escherichia coli* JCM1649, Ent.C, *Enterobacter cloacae* JCM1232; K.P., *Klebsiella pneumoniae* JCM1662; B.F., *Bacteroides fragilis* SMUM2275; H.I., *Haemophilus influenzae* ATCC33391; C.A., *Candida albicans* NBRC1594; C.T., *Candida tropicalis* NBRC1400; C.G., *Candida glabrata* NBRC0622; C.K., *Candida krusei* NBRC0011. GPC, Gram-positive cocci; GNR, Gram-negative rods.

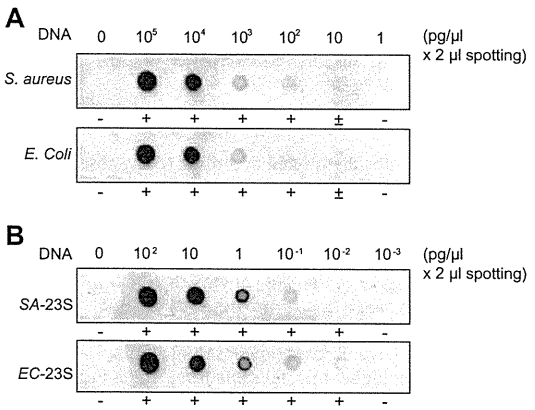

Fig. 4. Sensitivity of the ISH test for detecting bacterial DNA by the dot-blot hybridization with GB-probe. (A) The sensitivity analysis for the detection of bacterial genomic DNA with the GB probe. Samples containing serially diluted genomic DNA of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100,000, 10,000, 1000, 100, 10 and 1 pg/μl). Two microliters of each sample was spotted onto the membrane, and these were used for the hybridization with the GB probe. The ISH test showed positive signals on the spots which contained more than 200 pg of genomic DNA, whereas a positive signal was barely detected on the spot which contained 20 pg of genomic DNA. (B) The sensitivity analysis for the detection of target DNA (*S. aureus* [SA-23S] and *E. coli* [EC-23S]-DNA) with the GB probe. Samples containing serially diluted DNA fragments that corresponded to the 23S rRNA genes of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared (final concentration; 100, 10, 1, 0.1, 0.01, and 0.001 pg/μl). Two microliters of each sample was spotted onto the membrane, and these were used for the detection of bacterial DNA by the GB probe. The GB probe showed positive signals on the spots which contained more than 0.02 pg of the target DNA.

"Patients and methods". Eleven of the 51 patients had SBP defined by PMN counts equal to or over 250/μl (SBP patients), and the remaining 40 patients with the PMN counts below 250/μl were defined as non-SBP patients. The baseline clinical

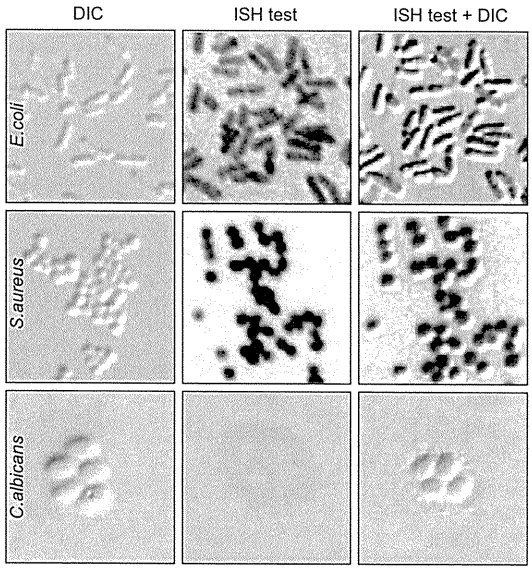

Fig. 5. *In situ* hybridization of the GB probe for the bacterial smear samples. In order to examine whether the ISH test can detect and visualize the genomic DNA of a single bacterial cell, smear samples of either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains were prepared. In addition, smear samples of a fungus (*C. albicans*: NBRC1594) were also prepared as negative control samples. We assessed the results of the ISH tests using the differential interference contrast microscope (DIC). The ISH tests succeeded in providing visualization of all smeared bacterial cells, thus suggesting that the GB probe can detect the small amount of bacterial DNA which is derived from a single bacterial cell.

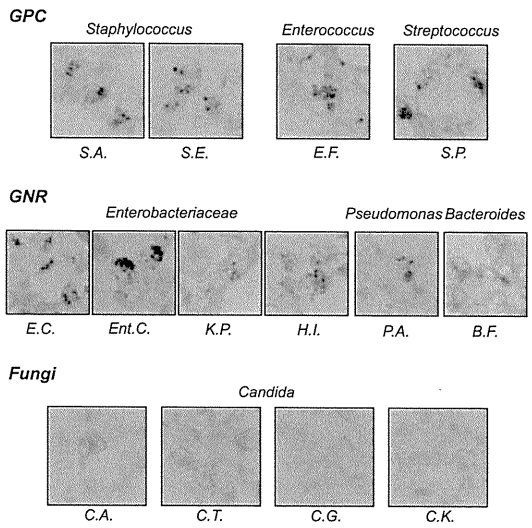

Fig. 6. Detection of the phagocytized bacterial DNA in leukocytes by the ISH method using the GB probe. Various bacterial strains were ingested by leukocytes *in vitro* according to the method described in "Patients and methods", and whether or not the GB probe could detect intracellular bacteria was determined. Both Gram-positive and Gram-negative strains were examined, and the ISH test was performed. Smear samples of leukocytes which had phagocytized fungi were used as negative control samples. Positive (purple brown) signals were observed in leukocytes (counterstained by fast green) which had phagocytized various Gram-positive (GPC) and Gram-negative (GNR) bacteria. However, no positive signal was observed in the leukocytes of intra-cellular fungal DNA (Fungi). The names of the bacterial or fungal strains are shown in the legend of Fig. 3. GPC, Gram-positive cocci; GNR, Gram-negative rods.

data of the groups distributed according to the absence or presence of SBP are shown in Table 3. The PMN counts of the SBP patients were significantly higher than those of the non-SBP patients, which is consistent with the diagnostic definition of SBP. The rate of variceal bleeding at the time of admission was significantly higher in the SBP patients than that in the non-SBP patients. The comparison of the other parameters analyzed did not show statistical significance, although there was a trend of more advanced liver disease (lower serum albumin level and prothrombin time; higher total bilirubin level, Child–Pugh score, and MELD score) in the patients of the SBP group. The SBP prevalence according to the etiology of cirrhosis was as follows: HBV: 0/6, HCV: 5/16, HBV + HCV: 0/1, non-viral: 6/28. Although there were no HBV-related cirrhotic patients who had SBP, the difference between the viral group (5/23) and the non-viral group (6/28) was not significant.

Hybridization of the new GB probe with bacterial genomic DNA

A previous study established five kinds of probes (designated SA, SE, PA, EF, and EK) and reported their specific detection of the intended bacterial genomic DNA. The present study attempted to generate a new probe, which was expected to hybridize with the genomic DNA sequences of 23S rRNA genes in global bacteria. At first, dot-blots were used to determine whether the newly developed GB probe could hybridize with the genomic DNA of various bacteria. As shown in Fig. 3, the GB probe detected all kinds of genomic DNA isolated from broad-spectrum bacterial strains, but did not react with the DNA of fungi, thus showing selective hybridization with the bacterial genomic sequences. In order to determine the lowest limit of the detectable bacterial DNA, serial dilutions of both whole genomic DNA and the target DNA for the genomic sequences of 23S rRNA were prepared and hybridized with the GB probe. As shown in Fig. 4A, the GB probe showed positive signals on the spots which contained over 200 pg of genomic DNA, and a weak signal was detected on the spot which contained 20 pg of genomic DNA. With regard to the detection of target DNA for 23S rRNA, the GB probe showed positive signals on the spots which contained over 0.02 pg of the target DNA (Fig. 4B). Therefore, the lowest amount of DNA detectable by the GB probe, was estimated to be about 20 pg of genomic DNA and about 0.02 pg of target DNA.

Furthermore, in order to determine the lowest limit of the detectable bacterial DNA, we examined whether the ISH test could detect and visualize the genomic DNA of a single bacterial cell. As shown in Fig. 5, the ISH test succeeded in the visualization of all smeared bacterial cells, thus suggesting that the GB probe can work in the detection of the small amount of bacterial DNA present in a single bacterial cell.

Next, the ISH method was used to determine whether the GB probe could detect intracellular bacterial DNA. As shown in Fig. 6,

Table 4. Bacterial strains detected by the GB probe.

Bacteria	genus	species	Strain
Actinobacteria	*Eggerthella*	*lenta*	JCM9979
	Corynebacterium	*diphtheriae*	JCM1310
		pseudodiphteritcum	JCM11665
		jeikeium	JCM9384
	Propionibacterium	*acnes*	JCM6425
	Micrococcus	*luteus*	JCM1464
Firmicutes	*Lactobacillus*	*fermentum*	JCM1173
		acidophilus	JCM1132
	Bacillus	*cereus*	IFO15305
	Staphylococcus	*aureus*	ATCC12600
		epidermidis	ATCC14990
	Enterococcus	*faecalis*	JCM5803
		faecium	JCM5804
		avium	JCM8722
	Streptococcus	*pneumoniae*	ATCC39938
		sanguinis	JCM5708
		pyogenes	JCM5674
		agalacticae	JCM5671
		salvarius	IFO13956
	Clostridium	*perfringens*	JCM1290
	Peptoniphilus	*asaccharolyticus*	JCM8143
Bacteroides	*Bacteroides*	*fragilis*	JCM11019
		ovatus	JCM5824
	Porphyromonas	*asaccharolytica*	JCM6326
Fusobacterium	*Fusobacterium*	*nucleatum*	JCM8532
		necrophorum	JCM3724
Proteobacteria α	*Brevundimonas*	*diminuta*	NBRC14213
Proteobacteria β	*Burkholderia*	*cepacia*	SMUM2242
	Achromobacter	*xylosoxidans*	JCM9659
Proteobacteria (γ)	*Pseudomonas*	*aeruginosa*	JCM5962
		fluorescens	JCM5963
		putida	JCM6157
	Acinetobacter	*calcoaceticus*	JCM6842
	Escherichia	*coli*	JCM1649
	Enterobactor	*cloacae*	JCM1232
		sakazakii	JCM1233
		aerogenes	JCM1235
		gergoviae	JCM1234
	Klebsiella	*pneumoniae*	JCM1662
		aerogenes	IFO13541
		oxytoca	SUMUM2071
	Raoultella	*terrigena*	IFO14941
	Haemophillus	*influenzae*	ATCC33391
	Serratia	*marcescens*	JCM1239
		liquefaciens	JCM1245
	Citrobacter	*koseri*	JCM1659
	Hafnia	*alvei*	JCM1666
	Edwerdsiella	*tarda*	JCM1656
	Proteus	*vulgaris*	IFO3851
		mirabilis	JCM1669
	Providencia	*rettgeri*	JCM1675
		alcalitaciens	JCM1673
		stuartii	ISTU683
	Morganella	*morganii*	JCM1672
	Salmonella	*enterica*	IFO12529
	Pantoea	*agglomerans*	JCM1236
	Kluyvera	*intermedia*	JCM1238
	Raoultella	*planticola*	IFO14939
	Stenotrophomonas	*maltophilia*	JCM1975

Data are expressed as the mean ± standard deviation or median (range).

positive signals were observed in cells which ingested various bacterial strains, but the GB probe did not react with cells that had phagocytized fungi. Therefore, positive signals detected by the GB probe were not due to non-specific hybridization, but were caused by the hybridization with genomic DNA of bacteria, suggesting the specific detection of intracellular bacterial genomic DNA. The GB probe was confirmed to hybridize with the genomic DNA of 59 bacterial strains (59 species of 36 genus) without exception (Table 4), thus suggesting that it was a new probe to detect global bacteria.

In order to examine whether the ISH test also work in fluid with lower concentration of leukocytes, serial dilutions (1000, 300, and 100/μl) of leukocytes were spiked with bacteria, and then leukocytes were collected to prepare smear samples. Using the experimental system of the *in vitro* phagocytization, the ISH test showed positive signals in all samples (Fig. 7), thus suggesting that the ISH test should work in the fluid with a lower concentration of leukocytes.

Preparation of ascitic leukocyte-smears and detection of bacterial DNA

The ISH tests against leukocytes samples isolated from blood samples used 5 ml of the peripheral blood samples for the tests. The current study used 100–200 ml of ascitic fluid samples, and leukocytes were collected by centrifugation, since the number of leukocytes in ascites is predicted to be less than one-tenth of that in blood samples. The average recovery rate of ascitic leukocytes was about 24.6%, and that was a sufficient number of cells for the investigation (data not shown). In addition, cellular shapes were well maintained after the centrifugation and re-suspension procedure, resulting in the successful preparation of smear samples.

Next, the ISH method was used to detect the bacterial DNA ingested in phagocytes. Smear samples of ascitic leukocytes were prepared according to the procedure described in "Patients and methods". As shown in Fig. 8, positive signals were detected in

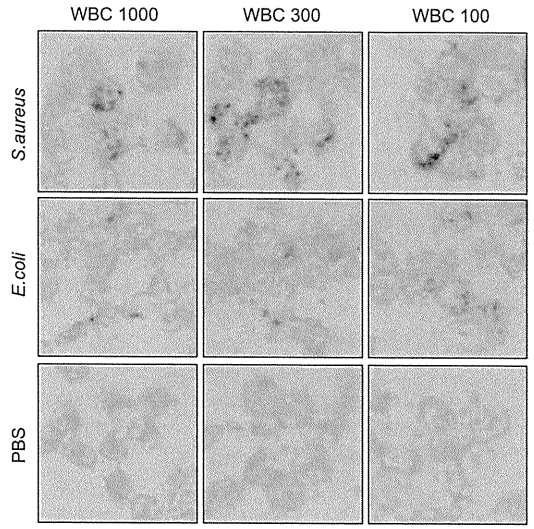

Fig. 7. *In vitro* phagocytosis of bacteria by low counts of WBCs, and the detection of bacterial DNA with the GB probe. In order to examine whether the ISH test works in ascitic fluid with a lower concentration of leukocytes, serial dilutions (1000, 300, and 100/μl) of leukocytes were spiked with either Gram-positive (*S. aureus*: ATCC12400) or Gram-negative (*E. coli*: JCM1649) bacterial strains, and then leukocytes were collected to prepare the smear samples. Using the same experimental system used for the *in vitro* phagocytization, the ISH test showed positive signals in all samples, thus suggesting that the ISH test can work in fluid samples with a lower concentration of leukocytes.

ascites-derived leukocytes of SBP cases, as well as they were observed in the peripheral leukocytes of septic patients in the previous reports [9,10].

The detailed results of the ISH tests and ascitic cultures in SBP patients are shown in Table 5. The previously developed probes

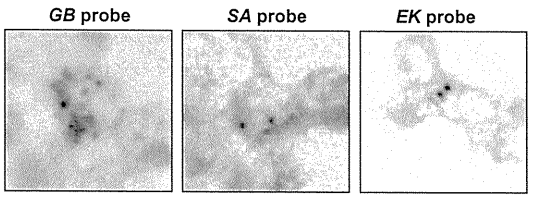

Fig. 8. Detection of the ingested bacterial DNA in ascitic leukocytes of SBP patients using the ISH method. ISH tests were performed to detect the bacterial genomic DNA in ascitic leukocytes obtained from cirrhotic patients. The procedure of ISH was based on the previously reported method with some modifications (see Fig. 2). Positive signals were detected in the ascites-derived leukocytes of SBP cases, as well as in the experimental studies shown in Fig. 6. Representative data of positive signals detected by the various ISH probes (GB probe, SA probe, and EK probe) are shown.

for specific pathogens (SA, SE, PA, EF, and EK) detected the bacterial genomic DNA in 4 of the samples from SBP cases (Nos. 1, 2, 4, and 9). In addition, positive signals were detected in 10 of the 11 cases when all available probes were used (including GB probe). Both the ISH test and culture showed positive results in the three cases (No. 1, 6, and 7). ISH and culture methods detected *S. aureus* in case No. 1. An ascitic culture revealed an infection of bacteria in cases No. 6 and No. 7, which were not identified by the five previously developed probes, but an infection could be successfully detected by the GB probe. Therefore, the results of the ISH test were consistent with the results of ascitic cultures in these three cases, suggesting the validity of the positive results obtained with the ISH method. In contrast, neither ISH tests nor ascitic cultures showed positive results in the 40 cirrhotic patients without SBP (Table 6). Therefore, when SBP was diagnosed at the threshold of 250 PMN count/μl with the ascitic fluid, the ISH test detected intra-cellular bacterial DNA in the SBP ascites with a high sensitivity (91%) and specificity (100%), despite the fact that the number of patients in the present study was small. The ISH tests with all probes (GB, SA, SE, PA, EF, and EK

Table 5. Results of *in situ* hybridization test for SBP patients.

Case No.	WBC (PMN) (cells/μl)	Results of ISH tests						Results of ascitic culture
		New probe	Previously established probes for specific pathogens					
		GB	SA	SE	PA	EF	EK	
1	5430 (4832)	+	+	-	-	-	-	MRSA
2	1400 (1141)	+	-	-	-	-	+	n.d.
3	1030 (991)	+	-	-	-	-	-	n.d.
4	1880 (1767)	+	-	-	-	-	+	n.d.
5	310 (281)	-	-	-	-	-	-	n.d.
6	7230 (6652)	+	-	-	-	-	-	*Serratia liquefaciens*
7	24,370 (20,593)	+	-	-	-	-	-	NF-GNR
8	5710 (4653)	+	-	-	-	-	-	n.d.
9	810 (535)	+	-	-	-	-	+	n.d.
10	3130 (2612)	+	-	-	-	-	-	n.d.
11	1380 (994)	+	-	-	-	-	-	n.d.

ISH, *in situ* hybridization; SBP, spontaneous bacterial peritonitis; PMN, polymorphonuclear neutropils; MRSA, Methicillin-resistant *S. aureus*; n.d.: not detected; NF-GNR: non-fermenting Gram-negative rod (unidentified: other than *Achromobacter xylosoxidans*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Chryseobacterium meningosepticum*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*).

Table 6. ISH test and culture of ascitic fluid in the cirrhotic patients.

	SBP (%)	Non-SBP (%)
ISH test (+)	10/11 (91)	0/40 (0)
ISH test (-)	1/11 (9)	40/40 (100)
Culture (+)	3/11 (27)	0/40 (0)
Culture (-)	8/11 (73)	40/40 (100)

probes) were performed simultaneously. Therefore, consistent with our previous reports [9,10], all of the ISH test results were obtained within one day.

Discussion

In the present study, a new ISH method to detect broad-spectrum bacteria was developed, and its utility to provide direct evidence of a bacterial infection in clinically defined SBP patients was evaluated. The principle of the method is to detect bacterial genomic DNA in the phagocytic leukocytes using ISH within 8 h, and this method was originally developed for the purpose of diagnosing sepsis. In addition to septicemia, a case report using the ISH test for the detection of bacterium in the peritoneal dialysis fluid was recently reported [17]. The current study showed that the ISH test is considered to be useful for obtaining early and direct evidence of a bacterial infection in SBP ascites.

While a delayed diagnosis of SBP often causes a fatal outcome, early and effective antibiotic treatment can improve the prognosis. Therefore, the diagnostic criteria of the complication focus on simplicity and rapidity, and the elevated PMN count in ascites is the gold standard for the diagnosis [2–4,8]. Although several days to weeks are required to reveal the casual bacteria by the ascitic cultures, the results are occasionally negative in patients with clinical symptoms of SBP. The amount of bacteria in the ascitic fluid of SBP patients is thought to be low even at the onset, and phagocytosis and digestion of bacteria by leukocytes should reduce the amount of proliferative and suspending bacteria in the ascitic fluid, thus leading to difficulty in identifying the pathogen. Previous studies assessed the clinical utility of several reactions induced by the host-immune responses of patients in order to develop a new method of SBP diagnosis. For example, reagent strips measuring leukocyte esterase activity have been reported to show a possible diagnostic utility of SBP [18,19]. In addition, the levels of several inflammatory mediators in ascitic fluids, such as tumor necrosis factor (TNF) and interleukin-6 (IL-6), are suggested to be associated with the severity of SBP [20,21]. However, these cytokines are generated by the host-reactions against inflammatory stimulations, thus these targets cannot directly demonstrate the bacterial infection of SBP. Moreover, the diagnostic accuracy of these methods has not yet been confirmed [2,22], although a new diagnostic method using the leukocyte esterase method was reported [23].

Recently, a PCR method has been reported to have a high sensitivity to detect the bacterial DNA in ascites [24,25]. However, a small amount of bacteria is suggested to invade into the peritoneal cavity of cirrhotic patients (so-called "bacterial translocation"), and the PCR method detects bacterial genomic DNA in about 40% of non-SBP ascitic samples [24,25]. Therefore, the higher sensitivity of the PCR method in comparison to the ISH test might lead to a lower diagnostic specificity. The present study attempted to detect ingested bacterial DNA by the ISH method. Although the sensitivity of the ISH test was 91%, no positive signal was detected in the non-SBP samples, thus indicating the highly specific (100%) detection of the bacterial infection. The lower detective sensitivity of ISH than the PCR method may, in turn, result in a higher specificity, because a fair amount of bacterial organisms causing clinical symptom(s) are required to show positive results based on the findings of ISH.

Previous reports have shown some advantages of the ISH method in the diagnosis of sepsis. At first, ISH showed high diagnostic sensitivity and the results were not affected by contamination [9]. In addition, ISH could be used for the diagnosis of septic patients receiving antibacterial treatment [11]. Consistent with the former advantage, the present data showed no false positive results in 40 non-SBP samples, suggesting that positive signals in SBP samples are not affected by contamination. The present study excluded patients treated with antibiotics, and, therefore, it was not possible to determine whether the latter advantage of ISH was also observed in SBP cases. However, there was one patient, who was diagnosed as SBP by the clinical manifestations and transferred to our hospital after the administration of antibiotics (one excluded case described in "Patients and methods"). The ISH test showed a positive result despite the low leukocyte count in the ascitic fluid (170/μl), implying the usefulness of ISH even after the initiation of the antibiotic treatment (Supplementary Table 1; patient A). These results suggest that several diagnostic merits of ISH in septic cases could be also applied to SBP patients. Since it is hypothesized that bacterial inflammation in ascites may induce episodes of systemic bacterial circulation, the ISH tests which were performed using the blood of SBP patients could provide useful information about the patients' status.

On the other hand, the ISH test fails to determine the *in vitro* susceptibility of causative organisms and this could be a problem with this method. However, the early detection of enterobacteria by the EK probe in the three cases of SBP (Nos. 2, 4, and 9) allowed for appropriate empirical treatments to be continued and, as a result, the patients recovered. Case No. 1 had a past history of severe acute cholecystitis, and had been treated with antibiotics for an extended period (over 2 weeks) until one month before the onset of SBP. This clinical information, in addition to the positive signals of SA probe (*S. aureus*), allowed the assumption of a Methicillin-resistant *S. aureus* infection. In the present study, we performed the ISH test with all probes (GB, SA, SE, EF, PA, and EK) simultaneously, and obtained positive signals of the SA probe within one day. One major advantage of the ISH test would be its orientation toward non-Gram-negative bacteria (including MRSA) which third-generation cephalosporins do not cover. Despite the lack of information on drug susceptibility, these cases suggested some clinical advantages of the ISH test, in comparison to the cases diagnosed only by the PMN counts without the information about the pathogen(s).

The ISH test showed positive signals in about 40% of the septic bloody samples in our previous study, whereas a higher sensitivity (91%) was obtained in the ascitic samples of the SBP patients in the present study. Since positive signals were detected in only 37% (4/11) cases by the previously established five probes (Table 5), the GB probe positively contributed to an increased sensitivity by detecting broad-spectrum bacteria in the present study. However, the GB probe cannot identify the characteristics of the pathogen, irrespective of the high sensitivity for the detection of bacterial DNA. In two SBP cases (Nos. 6 and 7), the

ingested bacterial DNA was detected by the ISH method, but the types of bacteria were only identified by the culture method. Although the present study showed the probable usefulness of ISH to detect the causative organisms of SBP, new probes for more kinds of bacteria are required, because such information regarding the pathogen is clinically important.

Previous studies reported the culture-positive rate of SBP ascites to be high, namely ranging between 72% and 90% of cases [3–5]. On the other hand, the rate of culture-positive cases was obviously low in the present study despite the fact that ascitic fluid cultures were performed by the standard (culture-bottle) method. However, this low culture-positive rate of SBP ascites has been also observed in several reports, with a proportion between 39% and 59% [7,14,26,27]. In addition, a recent report mentioned that positive results of ascitic culture are obtained in about only 40% of SBP cases [28]. Fernandez *et al.* showed low culture-positive rate (39%: 54/138), and they suggested that the low rate probably depended on an earlier diagnosis of the infection [7]. The current results may also reflect the earlier diagnosis, although that does not fully explain the low positive rate of cultures. A clinical trial with a larger scale should be performed to evaluate this new method in detail.

In the present study, we did not determine whether the ISH test works in bacterascites (positive bacterial culture without an elevated PMN count), because there were no patients who met the criteria for this conditions. However, our experimental studies suggested that ingested bacterial DNA can be detected even in fluid samples with a low count of leukocytes. Indeed, as described above, the ISH test showed a positive result in one patient who was treated with antibiotics for clinical symptoms of SBP, although the count of ascitic leukocytes was low. In contrast, when we investigated three cirrhotic patients with peritonitis carcinomatosa who showed high counts of ascitic leukocytes, no positive signal was observed (Supplementary Table 1; patients B, C, and D). Therefore, we think that the results of the ISH test should provide some clinical advantages in comparison to the cases diagnosed by the PMN counts alone. Since the ISH test detects phagocytized bacteria in SBP ascites, the results may, therefore, be independent of the leukocyte count. Although our preliminary results suggest that the ISH method is sensitive in cases with a low number of PMN, it remains to be demonstrated, in another study, that our technique is highly sensitive in SPB cases with PMN ranging from 250 to 1000/μl.

In summary, SBP is an infectious disease that is often only diagnosed by the PMN count without detecting the causal bacteria. The present study demonstrated the efficacy of a new probe to detect the DNA of global bacteria and showed the utility of the ISH test for providing early evidence of a bacterial infection in SBP ascites. The ISH test may, therefore, be a novel and effective approach for the management of SBP.

Conflict of interest

S.I. and A.M. are employees of Fuso Pharmaceutical Industries. The remaining authors have no conflicts to disclose.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.06.025.

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