Amplification of bacterial genomic DNA from all ascitic fluids with a highly sensitive polymerase chain reaction

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Abstract. Due to varying positive rates of polymerase chain reaction (PCR) amplification, interpretation of conventional PCR results for non-infectious ascites remains problematic. The present study developed a highly sensitive PCR protocol and investigated the positive rate of PCR for the 16S ribosomal (r)RNA gene in non-infectious ascites. Following the design of a new PCR primer pair for the 16S rRNA gene (800F and 1400R), the sequences of PCR products were analyzed and the lower limit for bacterial DNA detection evaluated. The positive rate of PCR for 16S rRNA gene in non-infectious ascites was also evaluated. PCR with the primer pair amplified the genomic DNA of 16S rRNA genes of major disease-causing bacterial strains. Additionally, PCR with this primer pair provided highly sensitive detection of bacterial genomic DNA (lower limit, 0.1 pg of template DNA). When DNA samples isolated from ascites were used, the 16S rRNA gene was amplified independently of the presence of bacterial infection. PCR products contained the genomic DNA fragments of multiple bacterial species. Bacterial genomic DNA can be amplified from all ascitic fluids using a highly sensitive PCR protocol. Careful attention is required to interpret the results based on simple amplification of 16S rRNA gene with conventional PCR.

Introduction

Spontaneous bacterial peritonitis (SBP) is one of the serious complications that can occur in cirrhotic patients and has high mortality and morbidity. Although the identification of pathogen(s) is essential for the management of infectious diseases, ascites fluid cultures often fail to provide positive results, even when using ascites samples from patients who develop clinical manifestations of SBP. Due to the necessity for early recognition and treatment, SBP is usually diagnosed on the basis of an absolute polymorphonuclear neutrophil (PMN) count in ascites with an optimal cut-off value of ≥250/mm³, even if the pathogen is not detected (1-4).

To overcome the difficulty experienced in obtaining direct evidence of bacterial infection in SBP ascites, an in-situ hybridization (ISH) method was recently developed and its potential clinical utility reported (5). Bacterial DNA detection and sequencing have been applied to the diagnosis of several infectious diseases. Molecular techniques can detect small amounts of bacterial DNA within a few h, and polymerase chain reaction (PCR)-based tests that target the bacterium-specific 16S-ribosomal (r)RNA gene would therefore provide a number of advantages. The highly conserved sequences of the gene allow broad-range detection of almost all bacterial species, while the hypervariable sequences can be used for pathogen-specific identification (6-9).

In the studies that have investigated ascites samples, PCR-based methods have demonstrated various positive rates and highly conserved sequences of 16S rRNA have been detected in ~30-60% of non-infectious ascites samples (10-14). However, with the detection of bacterial DNA in non-infectious clinical samples, serious criticisms of the contamination of PCR systems with bacterial DNA have been made (15-18). For instance, commercially available DNA polymerases can be contaminated with bacterial DNA, possibly as the products are generated as recombinant proteins in bacterial cells (15,16). Other commercial products including the reaction tubes for PCR analysis have also been reported to contain contaminating DNA fragments (17,18). However, if the amplification of 16S rRNA gene is caused by contamination in these commercially available products, bacterial DNA should be amplified in all
the samples tested. Previous studies have demonstrated that conventional PCR detects bacterial genomic DNA in ~30–60% of the non-SBP ascites samples tested (10-14). Therefore, it is also suggested that amplification of the 16S rRNA gene may reflect early detection of bacterial translocation in cirrhotic ascites.

Since previous studies have reported varying positive rates for PCR amplification, interpretation of PCR-based detection of the 16S rRNA gene in non-infectious ascites remains problematic. The present study developed a novel, highly sensitive PCR protocol and analyzed the amplification obtained using conventional PCR for the highly conserved sequences of the 16S rRNA gene.

**Patients and methods**

**Study population.** Cirrhotic patients with ascites who were admitted to Hyogo College of Medicine (Nishinomiya, Japan) between January 2010 and April 2013 were included in the present study. The study protocol conformed to the ethical guidelines of the 1975 Helsinki declaration and patients who agreed to the research use of ascites were enrolled following their informed consent. Cirrhosis was diagnosed on the basis of the histological results, clinical (laboratory or imaging) data, or both. Patients with any intra-abdominal, surgically treatable source of infection were excluded. Patients who received antibiotic treatment and patients with peritonitis carcinomatosa were also excluded from the analysis. The present study was approved by the Ethics Committee/Institutional Review Board of the Hyogo College of Medicine.

**Paracentesis.** Cirrhotic patients underwent diagnostic paracentesis under aseptic conditions using standard procedures for evaluation of the presence or absence of SBP. The routine biochemical variables and PMN count of the ascitic fluid were investigated. Ascites samples with a high PMN count (≥250/µl) and low PMN count (<250/µl) were considered as SBP ascites and non-SBP ascites, respectively. Blood samples were also collected to perform routine clinical studies.

**DNA extraction.** Genomic DNA was isolated from the bacterial strains according to previously reported methods (5). The following bacterial strains were obtained from Microbe Division/Japan Collection of Microorganisms RIKEN BioResource Research Center (Tsukuba, Ibaraki, Japan): *Escherichia coli* (cat. no. JCM1649), *Klebsiella pneumoniae* subsp. *pneumoniae* (cat. no. JCM1662), *Enterobacter cloacae* subsp. *cloacae* (cat. no. JCM1232), *Pseudomonas aeruginosa* (cat. no. JCM5962), *Bacteroides fragilis* (cat. no. JCM11019), *Enterococcus faecalis* (cat. no. JCM5803), *Enterococcus faecium* (cat. no. JCM5804), *Streptococcus pyogenes* (cat. no. JCM5674) and *Streptococcus agalactiae* (cat. no. JCM5671). The following bacterial strains were obtained from American Type Culture Collection, (Manassas, VA, USA): *Staphylococcus aureus* (cat. no. ATCC12600), *Staphylococcus epidermidis* (cat. no. ATCC14990) and *Streptococcus pneumoniae* (cat. no. ATCC39938). DNA was isolated from ascitic fluids according to the methods described by Such et al (10). In brief, 200 µl of an ascites sample was treated with an enzyme (lysozyme/proteinase K) containing buffer for 2 h, and DNA was extracted using QIAamp DNA Mini kit; Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols (10).

**Detection of bacterial DNA and DNA sequencing.** To establish a new PCR protocol, the highly conserved sequences of the 16S rRNA gene were analyzed. In the 16S rRNA gene (1,500 bp), it was identified that the sequences at positions 9, 350, 500, 800, 1,100 and 1,400 were highly conserved. To increase the sensitivity of the PCR, various primer candidates whose sequences were GC-rich in the 3'-position were evaluated. These DNA sequence-related analyses were performed with commercially available software (DNASTAR Lasergene, Ver.7.1; DNASTAR, Inc., Madison, WI, USA). The primer pair 5'-CAAACAGGATTAGATACCCCTGGTGATGCC-3' and 5'-TGTTGTACAAGGGCCCGGGAACGTATTACACC-3' was designed on the basis of its specific amplification of the 16S rRNA gene (800F-1400R; Fig. 1). Since two additional potential primer pairs [9F-500R: (5'‑AGATTTGATCCTGCGTCT AGGATGAAACGT‑3' and 5'‑TATTTACCGGCGTGTCG CACGGATTTAGC‑3') and 350F-1100R: (5'‑AGATTTTGA TCCTGCGTCAGATGAACGCT‑3' and 5'‑TATTTACCGGCGTGTCG TCACGGGATTTAGC‑3')] failed in providing a specific amplification of 16S rRNA gene (data not shown), we used the primer pair shown in Fig. 1. The fragments of the 16S rRNA gene were amplified with a Gene Amp PCR system 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using the primer pair with the following conditions: 94°C for 30 sec, 71°C or 55°C for 30 sec and 72°C for 30 sec for genomic DNA samples derived from bacterial strains; and 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30°C for ascites-derived DNA. PCR conditions, including tested DNA templates and reaction cycles were determined according to the methods described by Such et al (10). The lower limit of detection of bacterial DNA was then determined. Bacterial genomic DNA was used at various concentrations (10, 1 and 0.1 pg) as templates for the PCR.

The present study used two types of DNA polymerases: AmpliTaQ Gold LD (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Prime STAR HS (Takara Bio, Inc., Otsu, Japan). All PCRs were performed according to the manufacturers' protocols. Commercially available RNase-free water (Takara Bio, Inc.) was used in all reactions. To obtain the DNA sequences of PCR products, the amplified products were purified and sequenced according to standard direct-sequencing techniques. Each sequence was subjected to analysis with the Basic Local Alignment Search Tool (BLAST) of GenBank to investigate the homology of the 16S rRNA gene sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Results**

**PCR primers for highly sensitive amplification of the 16S rRNA gene.** Among the three potential primer pairs that corresponded to the conserved sequences of the bacterial 16s rRNA gene, the primer pair described in the 'Patients and methods' section was successfully used for the amplification of DNA fragments from multiple bacterial strains and whether the amplified PCR products were consistent with the fragments of 16S rRNA gene was investigated. Although the DNA
sequences were slightly different from each other, the amplified PCR products had sequences that were highly homologous to the 16S rRNA gene (Fig. 2). When 10 pg of bacterial DNA, which has been reported as the lower limit of detection for the conventional PCR method (10), was used, the 16S rRNA genes of major disease-causing bacterial strains were amplified (Fig. 3). In addition, amplification of the 16S rRNA target gene was confirmed for 59 bacterial strains that were used in our previous study (5) (data not shown).

Subsequently, serially diluted bacterial DNA solutions were used and the lower limit of bacterial DNA concentration for detection of the 16S rRNA gene with PCR. Evident bands were obtained with just 0.1 pg of each bacterial DNA template (Fig. 4).

Figure 1. PCR primers corresponding to conserved sequences for multiple bacterial genes. The 800F-primer is a forward primer corresponding to conserved sequences around base pair positions 800. The 1400R-primer is a reverse primer corresponding to conserved sequences around base pair positions 1400 (circle: one base mismatch). PCR, polymerase chain reaction.

Figure 2. Sequencing results for PCR products. PCR, polymerase chain reaction.
Detection of bacterial DNA from non-SBP ascitic fluids. Sensitive PCR amplification of the 16S rRNA gene was established and then it was investigated whether the gene could be detected in non-infectious ascites. A total of 24 cirrhotic patients had sterile (non-infectious) ascites as defined in the 'Patients and methods' section. The baseline clinical characteristics of the patients with non-infectious ascitic fluids are shown in Table I. All patients had cirrhotic livers, and the Child-Pugh score was B in 7 patients and C in 17 patients. A total of 12 patients were diagnosed with non-viral cirrhosis and 12 with hepatitis B or C viral infection. The remaining 12 patients had non-viral cirrhosis that was associated with various liver diseases, including alcoholism, autoimmune hepatitis, primary biliary cholangitis and cryptogenic hepatitis (Table I).

Although the conditions used for PCR in the present study allowed for highly sensitive amplification of the 16S rRNA gene, it was further investigated if conditions could be established for obtaining complete amplification of the gene from non-infectious ascites. When the results of the PCRs were carefully analyzed, the possibility that a PCR product was present in the negative control sample was noted, although the signal was very weak and uncertain (Fig. 3, lane N). To increase the sensitivity of the PCR, another DNA polymerase with higher efficacy of amplification was used (Prime STAR HS) and the annealing temperature selected as 55˚C. It was confirmed that the 16S rRNA target gene was more evidently amplified with the 0.1 pg of the bacterial DNA template under this condition (Fig. 5) compared with the results in Fig. 4. It was then attempted to detect the 16S rRNA target gene from non-infectious ascites. On doing so, it was identified that the PCR products were amplified from all 24 non-SBP samples (Fig. 6). All PCR products had homologous sequences to the 16S rRNA gene; however, multi-peak signals were observed at numerous sequence points, and identification of specific pathogens was difficult, suggesting that each PCR product contained the genomic DNA fragments of polymicroorganisms (Fig. 7). These results suggested that sensitive amplification of the 16S rRNA gene was achieved with our PCR protocol and that bacterial DNA can be amplified from all samples independently of spontaneous bacterial infection or bacterial translocation in the abdominal cavity, since these conditions are considered to be a monobacterial infection in ascitic fluid (1-4).

Discussion

SBP is an infectious disease that develops in cirrhotic patients with ascites. Bacterial culture often fails to detect the pathogen. It is known that the 16S rRNA gene is present in multiple copies in the genomes of bacterial pathogens and numerous bacterial species contain up to 7 copies of the gene (19). The presence of multiple copies can increase the possibility of detecting small numbers of pathogens, compared with assays performed for a single-copy gene. Therefore, PCR amplification of the bacterium-specific 16S rRNA gene is a useful method for investigating a broad range of bacterial species. However, it is unclear whether the PCR-based detection of the 16S rRNA gene is useful for determining the causative pathogen. A major problem is that the 16S rRNA gene can be amplified not only in SBP ascites but also in non-SBP sterile ascites, which makes it difficult to determine the clinical significance of this method.

Numerous commercially available recombinant DNA polymerases are generated in bacterial cells, and concerns about the presence of bacterial DNA in experimental items...
Fig. 7 may detect small amounts of contaminating bacterial genomic contamination has been established (14). Therefore, it has been also suggested that amplification of the 16S rRNA gene is associated with early detection of bacterial translocation in cirrhotic ascites, since a small number of bacteria are presumed to invade the intra-peritoneal cavity of cirrhotic patients with ascites via several pathways (24,25). Conversely, it has also been reported that PCR detection of bacterial DNA in non-infectious ascites is not directly associated with the development of SBP (26), and the clinical implications of detecting the 16S rRNA gene with PCR remain unclear.

The present study attempted to amplify the 16S rRNA gene in non-infectious ascites with newly-established conditions for PCR. Using this PCR protocol, a positive band could be obtained with 0.1 pg of bacterial DNA templates. This limit is 100 times more sensitive than the previously reported PCR protocols, whose lower limits of bacterial DNA templates were ~10 pg (10,27,28). However, difficulty was experienced in determining the bacterial species with DNA sequencing due to the possible presence of DNA fragments corresponding to multiple microbial species (Fig. 7). Soriano et al (14) studied 20 non-infectious ascites samples and could amplify the 16S rRNA gene in 12 samples. However, they succeeded in definitive bacterial identification only in 6 cases. They mentioned that the reason for the failure of the sequencing reaction could be low initial DNA concentration or the use of a mixture of amplification products that corresponded to multiple bacterial species. Tilburg et al (18) reported the probable contamination of a commercially available PCR Master Mix with bacterial DNA. They mentioned that the contamination was most likely caused by the use of compounds of animal origin due to the asymptomatic presence of several microorganisms in animals. As described above, although several studies have aimed to avoid bacterial DNA contamination of DNA polymerase (20-23), complete eradication of bacterial DNA is thought to be very difficult (16). The present study consistently demonstrated that a simple conventional PCR targeting the 16S rRNA gene invites criticism with respect to the identification of the causative pathogen.

To confirm the results of the present study, PCR was repeatedly performed, including using different batch numbers of PCR reagents, 16S rRNA genes were amplified from all non-SBP samples. The results of the present study may reflect contamination of commercially available PCR systems with multiple bacterial species. The possibility that the PCR products reflect the presence of bacterial DNA due to bacterial translocation cannot be dismissed, but, taking into account previous studies that demonstrated the risk of contamination, the contaminating bacterial DNA fragments would be considered to be mainly responsible for the reproducible and complete amplification of bacterial DNA. It is therefore suggested that contamination with bacterial DNA would be a commonly observed inevitable problem when using highly sensitive conventional PCR to identify the pathogen.

Table I. Characteristics of the patients.

| Parameter                        | Median value |
|----------------------------------|--------------|
| Age (years)                      | 59 (50-82)   |
| Sex                              |              |
| Male                             | 17           |
| Female                           | 7            |
| Etiology                         |              |
| HBV                              | 4            |
| HCV                              | 8            |
| Non-viral                        | 12           |
| Child-Pugh classification        |              |
| A                                | 0            |
| B                                | 7            |
| C                                | 17           |
| Total bilirubin (mg/dl)          | 2.3 (0.5-28.3)|
| Albumin (g/dl)                   | 2.7 (2.0-4.3)|
| PT-INR                           | 1.35 (1.02-2.30)|
| Hepatocellular carcinoma         |              |
| Present                          | 6            |
| Absent                           | 18           |
| PMN count of ascites (cells/µl)  | 10 (1-168)   |

HBV, hepatitis B; HCV, hepatitis C; PT-INR, prothrombin time-international normalized ratio; PMN, polymorphonuclear leukocytes. Numerical variables were expressed as the median values (range, min-max value).

Figure 5. Amplification of 16S rRNA genes with higher efficacy of amplification. When DNA polymerase with higher efficacy of amplification (Prime STAR HS) was used and the annealing temperature of 55˚C selected, the 16S rRNA target gene was clearly amplified from the DNA template of Escherichia coli. rRNA, ribosomal RNA.

DNA. However, if the amplified 16S rRNA gene from genomic DNA products of the clinical samples really represents the contaminated DNA, all PCRs should demonstrate positive results independent of bacterial infection. Nevertheless, previous studies have demonstrated that the PCR method can amplify 16S rRNA gene in fewer than 60% of sterile ascites samples (10-14). Therefore, it has been also suggested that amplification of the 16S rRNA gene invites criticism with respect to the identifica...
Recently, in addition to the efforts on eliminating contaminating bacterial DNA, several new approaches have been reported to succeed in providing a clinical significance of 16S rRNA gene amplification (29-32). For instance, the amount of 16S rRNA gene has been shown to be associated with the prognosis of cirrhotic patients (29,30). Additionally, advanced PCR-based methods for identifying bacterial pathogens, which cause SBP, have been also reported; excellent results were obtained with these assays, which should provide an improved approach for detecting pathogens (31,32). To the best of the authors' knowledge, the present study is the first report regarding the 100% amplification of 16S rRNA gene from non-infectious ascitic samples by a conventional PCR method. Its results suggest limitations of the simple PCR amplification and support the importance of the abovementioned recent superior techniques (29-32).

In conclusion, although recent advanced methods should demonstrate a clinical relevance, it is difficult to accurately detect the bacterial translocation in cirrhotic ascites with only a simple conventional PCR targeting the 16S rRNA gene. Careful attention is required to interpret the results based on simple amplification of 16S rRNA gene with conventional PCR.

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Availability of data and materials
The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions
HE contributed to the study design, performed the experiments, analyzed the data and drafted the manuscript. SI-I and AM performed the experiments and edited the manuscript. NA, TT and HN contributed to the sample collection, data acquisition and data analysis. YI, YS, RT, NK, KH, CN, TN, KY, YM, NIs, YY,
AI and HI contributed to sample collection and data acquisition. SN contributed to the study design, analyzed the data and edited the manuscript. All authors were involved in the manuscript revision and approved the final version of the manuscript.

Ethics approval and consent to participate

The study protocol conformed to the ethical guidelines of the 1975 Helsinki declaration and patients who agreed to the research use of ascites were enrolled following their informed consent. The present study was approved by the Ethics Committee/Institutional Review Board of Hyogo College of Medicine.

Consent for publication

Patients gave informed written consent.

Competing interests

The authors declare that they have no competing interests.

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