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Identification of Bacterial Pathogens in Ascitic Fluids from Patients with Suspected Spontaneous Bacterial Peritonitis by Use of Broad-Range PCR (16S PCR) Coupled with High-Resolution Melt Analysis

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Spontaneous bacterial peritonitis (SBP) can be a severe complication occurring in patients with cirrhosis and ascites, with associated mortality often as high as 40%. Traditional diagnostics for SBP rely on culture techniques for proper diagnosis, although recent reports suggest that the presence of bacterial DNA in peritoneal fluid in patients with cirrhosis and ascites is an indicator of SBP. A previously published broad-range PCR (16S PCR) coupled with high-resolution melt analysis (HRMA) was compared with standard culture techniques for diagnosis of SBP in 106 peritoneal fluid samples from patients with suspected SBP. The sensitivity and specificity for 16S PCR for detecting eubacterial DNA compared with those of standard culture techniques were 100% (17/17) and 91.5% (85/89), respectively. Overall, HRMA concordance with species identification was 70.6% (12/17), although the 5 samples that were discordant at the species level were SBP resulting from a polymicrobial infection, and species-level identification for polymicrobial infections is outside the capability of HRMA. Both the broad-range 16S PCR and HRMA analysis provide useful diagnostic adjunctive assays for clinicians in detecting and identifying pathogens responsible for SBP.

Spontaneous bacterial peritonitis (SBP) is a common and potentially fatal bacterial infection in patients with cirrhosis and ascites, occurring in 10 to 30% of patients, with in-hospital mortality rates ranging from 20 to 30% (1, 2, 6–8, 12). It is secondary to impaired humoral and cellular immune responses that result in indirect intestinal bacterial translocation into the ascitic fluid (1, 2, 6–8, 16, 20). SBP is also associated with a poor long-term prognosis for patients, as mortality rates can reach 50 to 70% at 1 year (2). Given that timely and appropriate antibiotic treatment can improve the clinical outcome, rapid and accurate diagnostic methods for early detection of eubacterial infection responsible for SBP and identification of the causative organisms involved could be particularly useful in acute care settings. Recent attention drawn to the changing microbial and resistance patterns attributed to the increasing use of antibiotic prophylaxis and invasive procedures in such patients further underscores the importance of identifying the causative pathogen to ensure adequate antibiotic coverage (13).

Current laboratory diagnosis of SBP is defined as ≥250 polymorphonuclear (PMN) cells/ml and a positive culture from ascitic fluid from the patient (1–13, 15–17, 19–22, 29). Unfortunately, the prolonged turnaround time (1 to 2 days) of culture limits its utility for directing antibiotic selection in acute care settings. Ascitic fluid culture has also been reported to be negative in approximately 20% of patients with clinical manifestations suggestive of SBP and an ascitic PMN count of >250, so-called culture-negative neutrocytic ascites. On the other hand, a low ascitic PMN count (<250) with positive culture can also occur in another SBP variant called bacterascites, or monomicrobial nonneutrocytic bacterascites. In either of the variant cases, empirical antibiotic treatment for presumed SBP is recommended.

Nucleic acid amplification tests (NAATs) have come to the forefront of infectious disease diagnostic development due to their higher sensitivity and specificity, culture growth independence, and rapid time to result compared to those of conventional culture methods. NAATs that target the bacterium-specific 16S rRNA gene can offer many advantages; the highly conserved sequences of the gene allow broad-range detection of almost any eubacterial species, while the hypervariable sequences can be exploited for species-level identification. Simultaneous detection of the 16S rRNA gene in serum and ascitic fluid in patients with culture-negative, nonneutrocytic ascites has been interpreted as a surrogate marker of bacterial translocation (21) and an independent predictor of 12-month mortality (29). Identification of the causative agent in ascitic fluid after 16S rRNA gene amplification has relied on either probe-based amplicon analysis, which limits testing to a finite number of anticipated pathogens, or sequencing, which is low throughput. We have previously combined 16S rRNA PCR (16S PCR) with high-resolution melt analysis (HRMA) for rapid broad-range detection and identification of bacterial pathogens (18, 24–28). HRMA offers a simple, low-cost, closed-tube approach to amplicon analysis with the capacity for single-nucleotide discrimination and easy integration with PCR analysis.

For this study, the performance of our 16S PCR-HRMA assay was evaluated against standard culture techniques to determine the sensitivity, specificity, and concordance with species identification in peritoneal fluid samples from prospectively enrolled patients with suspected SBP.

MATERIALS AND METHODS

Study population, setting, and sampling. From May 2009 to March 2010, waste peritoneal fluid samples (1-ml volume) were collected from

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106 patients with suspected SBP at the Johns Hopkins Hospital Emergency Department (ED) under a study protocol approved by the Johns Hopkins University Institutional Review Board. Patients in the study population were of mixed age and race, and all patients met clinical criteria for suspicion of SBP, including fever, ascites, and abdominal pain. Peritoneal fluid sampling was performed utilizing standard and universal precautions to ensure that a sterile sample was collected before delivery to the laboratory for peritoneal fluid culture and nucleic acid extraction and amplification. PMN data were available retrospectively and were obtained qualitatively. PMN counts were defined as none, rare or few, moderate, and many. All patient samples collected under this protocol were sent for clinical microbiological diagnostics to identify bacterial pathogens in the peritoneal fluid from these patients under standard of care practices.

Peritoneal fluid culture and microbiological testing. Peritoneal fluid culture was performed utilizing BD Bactec medium (Becton, Dickinson, Sparks, MD) and the BacTec blood culture system (Becton, Dickinson, Sparks, MD) and under standard clinical care practices. Further microbiologic testing was performed for organism identification, including Gram stain, MIC testing, and other standard techniques, such as coagulase and oxidase testing.

Nucleic acid extraction from peritoneal fluid. Bacterial nucleic acid was extracted from 1 ml of peritoneal fluid by the addition of 100 μl of lysis buffer I from the Roche MagNA Pure LC DNA isolation kit I (Roche Diagnostics, Indianapolis, IN), followed by a 15-min incubation at room temperature (25°C). Samples were centrifuged at ≥13,200 rpm, and the pellet was resuspended in 50 μl of filtered (Microcon Ultracel YM-100 filters; Millipore, Bedford, MA), DNase I (Invitrogen, Carlsbad, CA)-treated water. Ten microliters of 0.5 μg/μl Lysostaphin (Sigma-Aldrich, St. Louis, MO) and 0.32 μg/μl Lysozyme (Sigma-Aldrich, St. Louis, MO) were added to the samples, followed by a 20-min incubation at 37°C. One microliter of 1× proteinase K (Roche Diagnostics, Indianapolis, IN) was added, followed by a 10-min incubation at 65°C. Samples were frozen at −80°C for 10 min, incubated at 95°C for 5 min, and sonicated for 10 min. Samples were briefly centrifuged at low speed to collect evaporate from the sonication step.

16S PCR. 16S PCRs were performed in 50-μl reaction mixtures and contained the following: 20 μl of template DNA or appropriate positive or negative control, 25 μl of ABI 2× Universal PCR mix (Applied Biosystems, Foster City, CA.), and 1.5 μl of each 67 μM forward (p891, 5′-TGGAGCATGTGGTTTAATTCGA-3′) and reverse (p1033, 5′-TGCGGGACTTAACCCAACA-3′) primers. This reaction mixture was filtered with Centricon YM-100 filters (Millipore, Bedford, MA) at ≥13,200 rpm for 10 min, followed by the addition of 1 μl of 1.25 U/μl AmpliTaq Gold LD (Applied Biosystems, Foster City, CA) and 1 μl of 10 μM Uniprobe (VIC 5′ VIC-CACGAGCTGACGACARCCATGCA-MGBNFQ 3′). Reactions were performed in triplicate and reaction mixtures amplified on an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA) under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min with the ROX reference turned on.

HRMA. Samples that were positive by 16S PCR were further analyzed by HRMA as previously described (24, 26). HRMA reactions were performed in a 10-μl reaction mixture volume with a 15-μl overlay of mineral oil (Sigma-Aldrich, St. Louis, MO) and were analyzed utilizing a LightScanner (Idaho Technologies, Salt Lake City, UT). HRMA reactions were performed in triplicate for each of three 16S RNA regions, V1, V3, and V6, utilizing the following: 4 μl of 2.5× LightScanner mix (Idaho Technologies, Salt Lake City, UT), 1 μl each of 1.5 μM forward and reverse primers for each region, V1, V3, and V6 (V1F, 5′-GYGGCGNACGGGTGAGTAA-3′; V1R, 5′-TTACCCCACCAACTAGC-3′; V3F, 5′-CCAGACTCCTACGGGAGGCAG-3′; V3R, 5′-CGTATTACCGCGGCTGCTG-3′; V6F, 5′-TGGAGCATGTGGTTTAATTCGA-3′; and V6R, 5′-AGCTGACGACANCCATGCA-3′) and 2 μl of LightScanner-grade water (Idaho Technologies, Salt Lake City, UT). Reaction mixtures were amplified in twin.tec semiskirted 96-well real-time PCR plates (Eppendorf, Hauppauge, NY) under the following conditions: 94°C for 30 s, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by two holds of 95°C for 30 s and 28°C for 30 s. LightScanner runs were performed based on the manufacturer's guidelines, and the HRMA database utilized for species identification was previously published by this group (24, 26). The combined HRMA melting curves generated from V1, V3, and V6 target regions for each sample were then compared to our existing database for signature melting curve profiles for species identification.

Discordant analysis. All samples received 16S PCR and HRMA utilizing V1, V3, and V6 primers. Samples that initially tested positive by 16S PCR but were negative by culture or unresolved by HRMA were subjected to further testing to determine additional characteristics of the samples, using specific Gram-typing probes for Gram-positive (5′ 6FAM-AGGTGGTGCATGGTTGTCGTCAGC-MGBNFQ 3′) and Gram-negative (5′VIC-ACAGGTGCTGCATGGCTGTCGTCAGCT-MGBNFQ 3′) bacterial organisms (17). Gram-typing reactions were performed only on samples where HRMA failed to provide species-level organism identification.

PCR reaction mixtures were composed and PCRs performed in the same manner as described above, with the only difference being the utilization of two separate probes.

Data analysis. For 16S PCR, positive results were defined as reactions having a cycle threshold (C_T) value of less than 37.5, the C_T cutoff determined by the appropriate negative controls, and an exponential increase in fluorescence above baseline. Positive amplification was confirmed through analysis of multicomponent data. A positive 16S PCR indicated the presence of bacterial DNA in the nucleic acids extracted from the ascitic fluid of a sample. For HRMA, positive results were defined as samples having melting curve profiles that matched existing curve profiles within the HRMA library as previously described (24, 26).

RESULTS

Cellular characteristics of ascitic fluid. Qualitative PMN data from ascitic fluid was obtained and demonstrated the following characteristics: 92.5% (98/106) of patient samples had no identifiable PMNs, 4.7% (5/106) had rare or few PMNs, and 2.8% (3/106) had a moderate amount to many PMNs in smears of their ascitic fluid.

Broad-range PCR and HRMA. 16S PCR identified 21/106 (19.8%) ascitic fluid samples as positive for eubacterial DNA, compared to 17 (16%) samples that were positive by culture. The overall sensitivity for detecting the presence of eubacterial DNA in an ascitic fluid sample and the specificity of the 16S PCR compared to that of standard microbiological culture techniques were 100% (17/17) and 91.5% (85/89), respectively. Specific details regarding the 4 culture-negative, 16S PCR-positive samples are found in Table 1, which describes the standard culture result for the sample, the 16S PCR result for the sample, the HRMA identification based on melt analysis and comparison of melting curve profiles with organisms within the HRMA library, and the results for discordant analysis with Gram-positive and Gram-negative probes when they were utilized. The 4 culture-negative, PCR-positive samples were initially identified as positive by 16S PCR, based on their C_T value below the negative cutoff; further analysis with HRMA identified three of the samples as polymicrobial and one as *Micrococcus luteus*. The discordant analysis of the three polymicrobial samples with Gram-positive and Gram-negative probes indicated two samples as positive by both Gram-positive and Gram-negative probes, while one tested positive by the Gram-negative probe only. Gram-typing reactions were not performed on the sample testing positive for *Micrococcus luteus* as it had a definitive, albeit incorrect, identification obtained by HRMA.

The concordance to the species level between HRMA and stan-

TABLE 1 Culture, PCR, and HRMA results for spontaneous bacterial peritonitis with Gram-typing probe analysis for polymicrobial samples

Sample	Culture result	16S PCR result	HRMA identification	Discordant analysis[f]
1	*Staphylococcus aureus*	Positive	*Staphylococcus aureus*	NA
2	MRSA	Positive	*Staphylococcus aureus*	NA
3	*Staphylococcus* sp.[e]	Positive	*Staphylococcus hominis*	NA
4	*Enterococcus faecalis*	Positive	*Enterococcus faecalis*	NA
5	*Pseudomonas aeruginosa*	Positive	*Pseudomonas aeruginosa*	NA
6	Coagulase-negative *Staphylococcus* sp.[e]	Positive	*Staphylococcus saprophyticus*	NA
7	Coagulase-negative *Staphylococcus* sp.[e]	Positive	*Staphylococcus lugdunensis*	NA
8	*Enterococcus faecium*	Positive	*Enterococcus faecium*	NA
9	Beta-hemolytic *Streptococcus* sp.[e]	Positive	*Streptococcus agalactiae*	NA
10	Coagulase-negative *Staphylococcus* sp.[e]	Positive	*Staphylococcus saprophyticus*	NA
11	*Serratia marcescens*	Positive	*Serratia marcescens*	NA
12	*Enterococcus faecalis*	Positive	*Enterococcus faecalis*	NA
13	*Klebsiella* sp. and *Enterococcus* sp.	Positive	Polymicrobial[a]	Positive by GP[c] and GN[d]
14	*Enterococcus* sp.	Positive	Unidentified[b]	Positive by GP
15	*Enterobacter aerogenes* and *Escherichia coli* and alpha-hemolytic *Streptococcus* sp.	Positive	Polymicrobial[a]	Positive by GP and GN
16	*Streptococcus anginosus* and *Staphylococcus intermedius* and *Streptococcus constellatus*	Positive	Polymicrobial[a]	Positive by GP
17	*Escherichia coli* and *Streptococcus viridans*	Positive	Polymicrobial[a]	Positive by GP and GN
18	Negative	Positive	Polymicrobial[a]	Positive by GP and GN
19	Negative	Positive	Polymicrobial[a]	Positive by GP and GN
20	Negative	Positive	Polymicrobial[a]	Positive by GN
21	Negative	Positive	*Micrococcus luteus*	NA

[a] Multiple melting curve profiles were generated, indicating a polymicrobial infection.
[b] A single melting curve profile was generated, but it was inconsistent with all organisms in the HRMA library.
[c] Gram-positive typing probe.
[d] Gram-negative typing probe.
[e] Further characterization was not possible due to lack of sample for sequencing analysis.
[f] NA, not applicable.

dard culture was 70.6% (12/17). Specific details regarding organism identification are shown in Table 1, including results for the 5 discordant samples. Of the 12/17 culture-positive samples correctly detected and identified by 16S PCR and HRMA, 50% (6/12) were *Staphylococcus* spp., 25% (3/12) were *Enterococcus* spp., 8.33% (1/12) were *Streptococcus* spp., 8.33% (1/12) were *Pseudomonas* spp., and 8.33% (1/12) were *Serratia* spp.

Discordant analysis. Of the five discordant samples, one infection was a rare *Enterococcus* sp., one was a mixed *Enterococcus* sp. and *Klebsiella* sp., and three were mixed infections containing *Staphylococcus* sp., *Streptococcus* sp., *Enterobacter aerogenes*, and *Escherichia coli* by culture. Specific details regarding discordant analysis for the 5 discordant samples are shown in Table 2. Gram-typing reactions were positive for both Gram-positive and Gram-negative typing probes in the three discordant samples that were identified by culture as having a mixture of Gram-positive and Gram-negative organisms. Additionally, Gram-typing reactions were positive for Gram-positive organisms only in the sample that contained the rare *Enterococcus* sp. The sample that contained a mixture of multiple *Staphylococcus* spp. and *Streptococcus* spp. by culture was positive by the Gram-positive typing probe only.

DISCUSSION

SBP can be a serious, fatal complication for individuals with ascites and cirrhosis, with high mortality and recurrence rates and poor long-term prognosis (2). Early identification of patients that are at high risk for the development of SBP has been shown to be critical for prognostic improvement (8). As mentioned above, although Gram-negative bacteria are predominantly responsible for SBP, with increasing antibiotic prophylaxis, exposure to hospital environment, and frequent invasive procedures, recent studies have shown a trend toward an increase in infections of Gram-positive bacteria, particularly enterococci, staphylococci, and streptococci (13, 23). Early recognition of SBP by detection of eubacterial presence in otherwise sterile ascitic fluid and identification of the causative organisms involved could influence clinical decisions regarding timely initiation of therapy and appropriate antibiotic selection to ensure sufficient coverage.

Although the PMN data obtained from patients enrolled in this study were qualitative, 92.5% (98/106) of patient samples had no identifiable PMNs, and of these, 13.3% (13/98) were positive by bacterial culture and 16S PCR, while HRMA correctly identified the bacterial organism present in 69.2% (9/13) of these samples. Additionally, qualitative PMN data from smears performed on ascitic fluid revealed that 4.7% (5/106) had rare or few PMNs, and of these samples, 60% (3/5) were culture and 16S PCR positive, with HRMA correctly identifying the bacterial species in 67% (2/3) of these samples. Lastly, among 3.8% (4/106) of samples having moderate numbers or many PMNs in ascitic smears, 1 (25%) was positive by culture and 16S PCR; the bacterial species in this sample was correctly identified by HRMA. As the PMN data obtained were qualitative as opposed to quantitative, it is difficult to directly compare this study to similar published reports (1, 3–5, 7–9, 11, 12, 16, 19–22, 29). Our 16S PCR was able to detect bacterial DNA in 100% (17/17) of culture-positive samples, and HRMA was able to correctly identify the bacterial species identified by culture in 70.6% (12/17) of positive samples in our study. The rate of positivity for bacterial DNA, 19.8% (21/106), detected from

ascitic fluid samples in our study is comparable with the rates in other reported studies (3, 4, 8, 9, 11, 12, 19–21, 29).

The overall sensitivity for detecting the presence of eubacterial DNA in an ascitic fluid sample and the specificity for the 16S PCR were high, at 100% (17/17) and 91.5% (85/89), respectively. Although the sensitivity of 16S PCR was high, at 100%, a positive 16S PCR result simply indicates the presence of bacterial infection in a sample and that the sample warrants further testing. Characterization of 16S PCR-positive results is always required to properly identify the causative organism behind a presumed case of SBP. These results are comparable to other diagnostic methods for bacterial DNA detection in peritoneal fluid evaluated in a fashion similar to the method utilized in this study (3–5, 8, 9, 12, 19–22). Other diagnostic methodologies for detecting the presence of bacterial DNA in peritoneal fluid also had sensitivities ranging from 75% to 100%, while the specificities for other reported methods were all high, at >90% (3–5, 8, 9, 12, 19–22).

Although the microbiological spectrum found in our patient samples generally follows patterns previously described in other studies (3), it is interesting that 83.3% (10/12) of samples with concordant culture and HRMA results were Gram-positive organisms while other reports have Gram-negative organisms identified as the most common isolates from ascites (3, 21). This higher prevalence of nosocomial infections follows the recent trend and may be due to the higher rate of exposure of our patient population to hospital environment. Among culture-positive samples with monomicrobial infections, the pathogen identified by HRMA was concordant with culture results in 92.3% (12/13) of cases. Our limited database of melt profiles for other clinically relevant enterococcal species besides *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus gallinarum* is a likely explanation for HRMA's inability to correctly identify the one sample that was positive for an unspecified enterococcal species by culture.

One of the current limitations of PCR-HRMA is its inability to resolve polymicrobial infections. Although HRMA did correctly identify all culture-positive samples with polymicrobial infections based on multiple dominant peaks in the derived melting curve, the ability to resolve the curve to identify the individual organisms involved is still lacking. Although HRMA was unable to resolve the polymicrobial status for 5 samples, discordant analysis utilizing Gram-typing probes correctly identified the composition of Gram-positive and Gram-negative organisms in each of these samples (Table 1). Although the species of bacteria were not identified, information regarding the composition of Gram types of organisms in ascitic fluid could be useful for adjusting antibiotic selection in the instance of polymicrobial infection from gastrointestinal sources in the ascitic fluid of patients. One potentially cost-effective assay algorithm is to combine Uniprobe with differentially labeled Gram-typing probes in the same initial 16S PCR, followed by HRMA for PCR-positive samples for rapid identification of monomicrobial infections. For investigation of suspected cases of polymicrobial infections by HRMA, more costly multiplex sequencing technologies, such as Pyrosequencing, would need to be incorporated to definitively resolve the composition of species involved (14).

It is possible that the 4 PCR-positive but culture-negative samples are false positives due to sample contamination, but the fact that the mean C_T value for these 4 samples was 28.4 $\pm$ 4.1 (mean $\pm$ standard deviation) would not be consistent with low-level DNA contamination and is suggestive of these samples being true positives. Additionally, no PMNs were recovered from these samples, which is in accordance with other findings from this study, as 76.5% (13/17) of true positives from this study had no PMNs visible by smear.

Ideally, future studies would include a large, sequential, prospective analysis where ascitic samples, blood counts, and more complete clinical information, including PMN counts, were obtained from the patients. Additionally, future studies would also include a direct comparison with another well-validated molecular method of bacterial DNA detection and identification, as well as standard microbiological culture diagnostics. Overall, 16S PCR coupled with HRMA could prove to be useful diagnostic adjunctive assays when determining suspected cases of SBP, particularly given the short total time for the assays, at 4 h, and the high sensitivity, specificity, and ability to perform species-level identification of bacterial pathogens.

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