ORIGINAL ARTICLE

Routine Use of 16S rRNA Gene Sequencing for Diagnosis of Culture-Negative Bacterial Infections in Body Fluids Other than Blood: Benefits in a Limited Resource Hospital

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ABSTRACT

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Background: *Culture-dependent methods sometimes fail to identify bacterial infections in samples of body fluids, particularly with prior administration of antimicrobial drugs. Nucleic acid amplification tests have become well-established diagnostic tools in microbiology laboratories, particularly 16S rRNA gene PCR (polymerase chain reaction) sequencing, as they can diagnose bacterial infections in patients.* **Objective:** *We evaluated the use of 16S rRNA gene PCR combined with DNA sequencing to detect and identify bacterial pathogens in cases suspected to have bacterial infection of body fluids with negative cultures.* **Methodology:** *Over one year, samples of cerebral spinal fluid (CSF), ascitic, pleural and synovial fluids were subjected to conventional analysis by culture methods, total leukocyte count (TLC), and chemical analysis.* **Results:** *Of the 304 culture-negative samples, 108 were accompanied with high TLC and predominant neutrophils. An 8.3% (9/108) 16S rRNA gene PCR-positivity rate was achieved for the samples, resulting in the identification of nine microorganisms. The protein content was significantly higher in the PCR-negative cases compared with the PCR-positive cases (82.2 ± 174 and 3.3 ± 2.1 respectively, p=0.01).* **Conclusion:** *Our results indicate that 16S rRNA gene PCR followed by amplicon sequencing appears to be a helpful diagnostic tool for the culture-negative ascitic and pleural fluid, and CSF samples, and the small sample size precluded us from exploring its applicability for use with synovial fluid samples.*
Abbreviations: *CFU, colony-forming unit; CSF, cerebrospinal fluid; LDH, lactate dehydrogenase; NAAT, nucleic acid amplification tool; PCR, polymerase chain reaction; PMNL, polymorphonuclear leukocyte; rRNA, ribosomal ribonucleic acid; SBP, spontaneous bacterial peritonitis; SD, standard deviation; TLC, total leukocyte count*

INTRODUCTION

Bacterial infections remain a significant cause of morbidity and mortality in humans.[1,2] Early detection of pathogens in clinical samples is an essential step for improved diagnosis and effective management of infections.[3,4] Conventional microbiological processing of clinical samples has traditionally relied on culturing microorganisms on various media and on the use of phenotypic identification methods.[1,5]

However, culture-dependent methods may fail to identify pathogenic organisms, particularly the slow-growing organisms, those with fastidious culture requirements, or those that are non-cultivable.[3] Prior administration of antimicrobial drugs before collection of clinical samples is another factor that interferes with pathogen isolation.[6,7]

Culture-negative bacterial infections can exist in up to 20% and 60% of cases of spontaneous bacterial peritonitis (SBP)[8] and empyema,[9] respectively. The low rate of pathogen isolation complicates the accurate diagnosis and selection of effective treatment for patients and highlights the crucial need for culture-independent diagnostic techniques.[8]

Nucleic acid amplification tests (NAATs) have become an essential component of the diagnostic workup in microbiology laboratories because of their high sensitivities and specificities, culture independence, and rapid turn around time compared to culture-based techniques.[5,8] NAATs include techniques that are pathogen-specific, multiplex, and broad-ranging.[10] The clinical application of pathogen-specific assays is narrowed by the diversity of pathogens potentially responsible for different types of infections. Multiplex real-time PCR (polymerase chain reaction) assays offer a potentially beneficial approach to the rapid detection of blood stream pathogens, as well as of clinical specimens other than blood. However, a clear limitation of multiplex real-time PCR assays is their failure to detect pathogens that are not incorporated in the targeted spectrum of the multiplex PCR.[3]

NAATs targeting conserved regions of bacterial genomes, particularly the 16S rRNA gene of bacteria, offer many advantages for bacterial identification when combined with DNA sequence analysis.[4] This PCR method, which is used mostly to diagnose culture-negative bacterial infections, potentially allows for the detection of cultivable and non-cultivable bacterial pathogens.[3] Because ribosomal RNA (rRNA) genes are universally present in bacteria, broad-range identification to the genus level is almost guaranteed,[11] and the hypervariable sequences can be used for identification to the species level.[8] Another advantage of broad-range 16S rRNA analysis in clinical practice relies on its ability to diagnose bacterial infections in non-selected patient populations, such as those with sepsis, endocarditis, central nervous system infections, spontaneous bacterial peritonitis, and orthopaedic infections.[3]

In the current study, we have used 16S rRNA gene PCR combined with sequence analysis to detect and identify bacterial pathogens in cases where bacterial infection of body fluids is suspected, but the cultures remain negative.

METHODOLOGY

Samples:

This study was conducted in Kasr Al Ainy Cairo University Hospital (Egypt) over one year (2018–2019). Different body fluid samples were submitted to the Microbiology Laboratory. The samples were obtained from pleural fluid, ascitic fluid, cerebrospinal fluid (CSF), and synovial fluid. Informed written consent was provided by each patient admitted to our hospital who participated in the study. Patients enrolled in the study approved the investigations and the treatment recommended by their physician. The research study was approved by the Clinical Pathology Department, Faculty of Medicine, Cairo University, and the study was performed on routine samples provided to the department's laboratory. No additional samples were extracted for research purposes. The total number of body fluid samples submitted over one year was 1494 samples distributed as follows: CSF (911), ascitic fluid (341), pleural fluid (225) and synovial fluid (17).

Cytological Examination and Chemical Analysis:

All samples were subjected to total leukocyte counting and examined for cell morphology (percentage of neutrophils and lymphocytes). Chemical analysis of the samples was performed to determine the levels of glucose, total protein, and lactate dehydrogenase.

Microbiological Methods:

All samples were cultured on blood agar, chocolate agar, and MacConkey's agar (Oxoid, Basingstoke, UK) for 24 hours at 37°C aerobically, in 5% CO_2, and anaerobically for 3 days. Samples were also cultured on brain heart infusion broth (Oxoid) and were sub-

cultured when they showed turbidity. Gram staining was done for all samples. The isolated organisms were identified using conventional microbiological procedures.[12] Of note, 304 out of the total number of submitted body fluid samples were culture-negative over one year. Samples were divided into two groups, group 1: with normal or high total leukocyte counts (TLCs) and a predominance of lymphocytes, and group 2: with high TLCs and a predominance of polymorphonuclear leukocytes (PMNLs).

Molecular Methods:

Molecular testing was done on 108 out of 304 body fluid samples, using selected criteria, which were samples showing no bacterial culture growth but high TLCs and a predominance of PMNLs, regardless of the gram stain examination or chemical analysis results. DNA was extracted using the Genomic BYF DNA Mini kit (iNtRON Biotechnology, Inc., Korea). The 16S rRNA gene was amplified using conventional PCR with forward primer 536f 5′-CAGCAGCCGCGGTAATAC-3′ and reverse primer RP2 5′-ACGGCACCTTGTTACGACTT-3′ (AccuOligo, Bioneer, Daejeon, Korea), as described previously.[13] Each analysis included a positive control prepared from nuclease-free water spiked with 10^4 colony-forming units cfu/mL of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) and negative control, which contained all PCR reagents except DNA. The positive and negative controls were included with every 10 test samples. PCR products were purified using a PureLink® PCR Purification Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. A BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used to sequence the PCR-amplified products from the PCR-positive cases, in accordance with the manufacturer's instructions. The sequenced products were resolved on a 3500 Genetic Analyzer (Applied Biosystems). Sequences were compared with those available in the GenBank database for 16S rRNA gene sequences (bacteria and archaea) using the basic local alignment search tool (BLAST, www.ncbi.nlm.nih.gov). Identification to the species level was defined as sequence similarity of ≥99% compared with that of the GenBank prototype strain sequence, while identification to the genus level was defined as sequence similarity of 97%–98.9% compared with that of the GenBank prototype strain sequence. Identification failure was defined as a sequence displaying < 97% sequence similarity with the sequences deposited in GenBank at the time of the analysis. Sequence similarity was interpreted according to the criteria reported previously.[14]

Statistical Methods:

All statistical calculations were conducted using the statistical package for social sciences (SPSS; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Data were statistically described in terms of the mean ± standard deviation (±SD), range, or frequencies (number of cases) and percentages, where appropriate. An independent sample t-test, for testing equality of means, was used to compare the PCR results with other sample variables. P values of ≤ 0.05 were considered to be statistically significant.

RESULTS

This study was conducted on different body fluid samples submitted to the Microbiology Laboratory at Kasr-Al Ainy University Hospital from 2018 to 2019. The sample characteristics (i.e., TLC, predominant cell morphology, and culture results) are shown in table 1.

Of the 108 samples that fulfilled the criteria chosen for molecular analysis, ascitic and pleural samples predominated at 36% and 35%, respectively. The mean TLC for the samples was $2521 \pm 5667/mm^3$ (median: 560, range 20,000–40,000), while PMNLs ranged from 50% to 100% (mean: 83.6% ± 11.8%). The distribution of the TLCs among the different body fluid samples is shown in table 2. Lactate dehydrogenase (LDH) was high in the samples, suggesting exudate formation in 60% of the cases, but these samples were PCR-negative. Glucose was low in 26% of the cases, suggesting a potential bacterial infection. The mean protein content was 49.6 ± 38.5 mg/dL (median 2.7), the glucose level was 101 ± 16.8 mg/dL, and the LDH level was 879.2 ± 444.7 IU/dL (median 241).

Table 1: An overview of the total body fluid samples examined in the microbiology lab

Sample type	Normal TLC or high TLC with predominance of lymphocytes	Positive culture samples with isolation of 1 or 2 organisms	Predominant microorganism isolated	Negative culture samples with high TLC with predominance of neutrophils
CSF (911)	588 (64.5%)	180 (19.75%)	*Pseudomonas aeruginosa* (36)	143 (15.75%)
Ascitic (341)	201(58.9%)	52 (15.24%)	*E.coli* (21)	88 (25.8%)
Pleural (225)	104 (46.22%)	59 (26.22%)	*Streptococcus pneumoniae* (11)	62 (27.5%)
Synovial (17)	2 (11.76%)	3 (17.64%)		11 (64.7%)

CSF, cerebrospinal fluid; TLC, total leukocyte count

Table 2: The distribution of the TLCs among the different body fluid samples

Samples (108)	Total leukocytic count No (%)			
	$<100/mm^3$	$100–1000/mm^3$	$1000–10\,000/mm^3$	$>10\,000/mm^3$
Ascitic (38)	4 (11%)	27 (71%)	5 (13%)	2 (5%)
Pleural (37)	2 (5.5%)	22 (59.5%)	7 (19%)	6 (16%)
CSF (29)	8 (27.5%)	10 (34.5%)	9 (31%)	2 (7%)
Synovial (4)	0	0	1 (25%)	3 (75%)

CSF, cerebrospinal fluid.

By PCR, 8.3% (9/108) of the samples were positive for bacterial 16S rRNA gene (pleural fluid samples, 4; ascitic fluid samples, 4; CSF, 1). DNA sequencing identified *Acinetobacter baumannii* (two cases), *Bacillus* spp., *Escherichia coli*, *Halomonas meridiana*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Streptococcus intermedius*, and *Streptococcus pneumoniae* (one of each), as shown in table 3

Table 3: Details of samples positive for 16S rRNA and sequencing

Patient ID	Sample type	TLC	PMNs %	Glucose	Protein	LDH	16S rRNA Sequencing	Sequence length bp	% Identity
4FL	Pleural	1950	70	121	4.7	120	*Salmonella enterica subsp enterica*	*988*	97%
6FL	Ascetic	150	60	122	1.6	94	*Acinetobacter baumannii*	*970*	99%
17FL	Pleural	12000	100	3	4.9	7929.3	*Strept intermedius*	*969*	99%
18CH	Pleural	650	75	134	2.9	265.8	*Strept pneumoniae*	*968*	99%
34CH	Pleural	110	70	135	2.3	241	*Acinetobacter baumannii*	*992*	94%
50ML	Ascetic	550	80	228	1.5	90	*E.coli*	*989*	98%
60FL	Ascetic	190	80	13	190	154	*Bacillus spp.*	*1195*	89%
106FL	Ascetic	>10,000	95	91	4.3	114	*Halomonas meridiana*	*970*	99%
65ML	CSF	2800	95	13	190	345	*K.pneumoniae*	*984*	96%

CSF, cerebrospinal fluid; LDH, lactate dehydrogenase; PMN, polymorphonuclear leukocyte; TLC, total leukocyte count.

No statistically significant differences were detected between the PCR-positive and PCR-negative samples regarding the leukocyte counts, PMNL percentages, glucose levels, and LDH levels. However, the protein content was higher in the PCR-negative cases than in the positive cases, a finding that was statistically significant (82.2 ± 174 mg/dL and 3.3 ± 2.1 mg/dL, respectively; p=0.01); the detailed data is shown in table 4.

Table 4: Relation between 16S PCR results and different parameters

	16S_PCR	No.	Mean	Independent sample t-test to test equality of means
TLC	0	67	2454.03	0.716
	1	5	3418	0.695
PMNs	0	69	83.6667	0.948
	1	6	83.3333	0.962
Glucose	0	30	91.8667	0.672
	1	1	121	-
Protein	0	36	82.2411	0.53
	1	2	3.25	0.01*
LDH	0	25	774.44	-
	1	0	.	-

*p value <0.05 is statistically significant
TLC: total leucocytic count
PMNs: polymorphnuclear cells
LDH: leucocyte dehydrogenase

DISCUSSION

Accurate identification of the organisms responsible for acute infectious diseases remains a big challenge for clinicians, especially when conventional microbiological workup remains negative. Appropriate, rapid, and reliable laboratory tests are necessary for the accurate diagnosis and management of infections in body fluids (e.g., ascitic, pleural, cerebrospinal, and synovial fluids). In the present study, out of the 1494 body fluid samples collected, 304 were found to be culture-negative over the one year encompassing 2018 to 2019, which represents 20% of the total number of samples submitted.

The additional value of 16S RNA gene PCR diagnostic screening of the culture-negative cases was the identification of 9 of 108 (8.3%) samples (from cases with high TLCs and predominant PMNLs).

This result is consistent with the finding of Shetty et al,[15] who stated that among 33 culture-negative samples, 3 samples (9.09%) were PCR positive. Yoo et al[7] stated that the diagnostic yield increased by 5.12% with the addition of 16S rRNA gene targeted sequencing.

In another study conducted by Grif et al[3], the use of universal 16S and 18S rRNA gene PCR followed by amplicon sequencing (SepsiTest) was evaluated against conventional culture on different body fluids. Their results showed that the PCR positivity rate (34.6%) was higher than that of bacterial culture (25.0%). They speculated that their findings resulted from the presence of fastidious microorganisms in the samples or the recent antimicrobial treatment of the patients. In their study, PCR detected 13 additional cases that were culture-negative, thereby enabling rapid identification of microorganisms and early administration of appropriate antimicrobial therapy.

In our study, the 16S RNA gene PCR followed by amplicon sequencing identified *Acinetobacter baumannii* (2 cases), *Bacillus* spp., *Escherichia coli*, *Halomonas meridiana*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Streptococcus intermedius*, and *Streptococcus pneumoniae* (one of each).

As regards these cases with high TLCs and negative cultures, although most of the bacteria detected by PCR are cultivable organisms, their culture-negative status may be attributed to the pretreatment of the patients with antibiotics, a result consistent with a previous report.[15–17]

In our study among, the 341 ascitic fluid samples subjected to ascitic fluid culture, 88 samples (25.8%) were culture-negative. Other studies have reported similar findings where ascitic fluid samples were found to be culture-negative for about 10%−60% of patients clinically diagnosed with SBP[18,19] In the present study, 92% of the ascitic fluid TLC counts ranged between 100 and 10000 cells/mm^3, which accords with the diagnosis of peritonitis. In the 16S rRNA gene-positive cases, sample (106FL) had a TLC of >1000 cells/mm^3 and sample (50ML) had a TLC of 550 cells/mm^3, while sample (6FL) and sample (60FL) had TLCs of 100–200 cells/mm^3.

Another study conducted by Enomoto et al[20] proposed PMN cutoff values for diagnosis of SBP and suggested that ascitic fluid samples with PMNs >250 cells/mm^3 have the highest sensitivity in the absence of an infection source, while those with PMN values of 500 cells/mm^3 have the highest specificity for confirming a diagnosis of SBP. In contrast, in another study[21] conducted on ascitic fluid samples, 16SrRNA gene PCR generated a high rate of positive cases (79%) in samples with PMNs ≤500 cells/mm^3, although only 3% of them were culture-positive. In that study, Krohn et al[21] successfully developed a way to enhance the bacterial DNA content of their samples before the nucleic acid extraction step, by using large sample volumes (up to 50 mL), degrading the human DNA present, and increasing the number of PCR cycles.

The present study reported that 62 out of 225 (27.5%) pleural fluid samples were culture–negative, and PCR identified a causative organism in 4 of 38 samples (10.5%). Higher culture-negative results were

found in another study conducted by Blaschke et al,[6] where 41 patients (65%) with pediatric parapneumonic empyema had no identifiable pathogen by the culture of either pleural fluid or blood. Of these 41 culture-negative patients, 30 (73%) had a pathogen identified by PCR of their pleural fluids.[6] Elsewhere, a study that analyzed purulent pleural fluid samples extracted from children reported that PCR alone could detect the microorganisms responsible for the infections in 14 samples (of 32) from clinically diagnosed patients; these microorganisms were predominantly *S. pneumoniae*, *S. pyogenes*, and anaerobic bacteria.[22]

Identification of the causative pathogen in osteoarticular infections is important for guiding the choice of appropriate antimicrobial therapies, but culture negativity in such infections is caused mainly by prior antimicrobial intake or the presence of fastidious microorganisms that require special media or prolonged incubation for growth. In the present study, 11 out of 17 (64.7%) synovial fluid sample cultures were negative. No samples were PCR-positive, but this might be attributable to the small number of submitted samples. Marin et al[23] conducted a study performed on synovial fluid samples, together with tissue samples, to diagnose prosthetic joint infections.[23] In their study, PCR generated a higher incidence of false-negative results than the cultures, with a false-negative culture rate of 22.15% and a false-positive rate for 16S RNA gene PCR of 32.86% for the cases; however, PCR had greater specificity than culture, as the positive predictive values for PCR and culture were 94.3% and 66.1%, respectively, and specificity values for PCR and culture were 97.8% and 81.2%, respectively.[23] The usefulness of broad-range 16S rRNA gene PCR was confirmed in another study,[24] which involved the diagnosis of an additional 141 culture-negative cases and included 3840 bone and joint samples, representing 6.1% of the total.

In our study, of the 911 CSF samples that were cultured, 180 (19.75%) were culture-positive, while 143 (15.75%) were culture-negative despite them having a predominance of neutrophils and elevated TLCs. From the culture-negative CSF samples, 26 were subjected to broad-range PCR, and an etiologic agent was identified in only one case. In a recent study conducted on CSF samples extracted from patients suspected to have central nervous system infections, 16S and 18S rRNA gene PCR detected bacteria and fungi in 65% of the meningitis cases, while culture and/or microscopic methods detected bacteria and fungi in 35% of the cases.[25] This study documented improvement in the diagnosis of infective meningitis through the use of PCR analysis over conventional microbiological methods.[25] However, a study by Kotilainen et al[26] found that PCR failed to detect the bacterial rRNA gene in one CSF sample, the culture of which yielded *Listeria monocytogenes*. Kotilainen et al[26] asserted that gram staining and bacterial culture should remain the cornerstone of meningitis diagnosis and they recommended that research efforts should be focused on the application of PCR with broad-range bacterial primers to improve the etiologic diagnosis of bacterial meningitis.[26]

In the present study, we only conducted PCR assays on the culture-negative samples to save resources because the PCR results would not affect the outcome in cases where positive-culture results were obtained. Additionally, in cases of polymicrobial infection, the PCR results will be misleading and sequencing will fail to identify a specific organism.[27]

The use of broad-range 16S rRNA gene PCR followed by sequencing was used previously in our laboratory with other samples. When this method was applied to blood samples from cases with bloodstream infections, this technique exhibited 86.25% sensitivity, 91.25% specificity, 76.67% positive predictive value, 95.22% negative predictive value, and an accuracy of 88.8%.[28] Also a 62.2% agreement was found between the culture results and the 16S rRNA gene PCR results when used on valve tissue samples in cases of infective endocarditis.[29]

CONCLUSION

16S rRNA gene PCR followed by amplicon sequencing appears to be a helpful diagnostic tool for culture-negative ascitic and pleural fluid and CSF infections and could not be used for effective analysis of synovial fluid infections because of the small sample size. Nevertheless, the method is a potentially useful additional tool for detecting certain cultivable or non-cultivable bacteria, especially in patients treated previously with antimicrobial drugs provided it is used in conjunction with bacterial culture. Further studies are needed to evaluate the cost-effectiveness of this method in the long term and the impact of this assay on the clinical outcomes of patients with culture-negative infections

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