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Detection of bacterial DNA in infected body fluids using 16S rRNA gene sequencing: Evaluation as a rapid diagnostic tool

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Abstract:

CONTEXT: Rapid detection of pathogens in infected body fluids will help in establishing an aetiological diagnosis, thereby facilitating specific therapy. Molecular methods have an advantage over conventional bacteriological techniques in particularly identifying slow-growing, fastidious or non-cultivable organisms.

AIM: The aim of this study is to evaluate the use of 16S rRNA polymerase chain reaction (PCR) for detecting bacterial pathogens in body fluids and compare with conventional culture methods.

SETTINGS AND DESIGN: This study was done at the Clinical Laboratory of the Department of Microbiology. This was a cross-sectional study design.

MATERIALS AND METHODS: A total of 100 consecutive samples which included synovial fluid, cerebrospinal fluid, ascitic fluid and pleural fluid received in the laboratory during the study period were subjected to PCR for 16S rRNA using specific primers and conventional culture by standard protocol. Samples which were positive for 16S rRNA were sequenced to identify the organism. Results of sequenced products were compared in terms of number of organisms, with culture isolates.

RESULTS: The detection rate of 16S rRNA PCR was at 13% as compared to culture at 3% ($P = 0.0009$). The diagnostic sensitivity and specificity of the PCR were 100% and 89.7%, respectively. The concordance of PCR and culture for both identical positive and negative samples was 90%.

CONCLUSIONS: The 16S rRNA PCR proved to be rapid method for detection of bacterial pathogens in body fluids. It may be a valuable tool in the diagnostic armamentarium for differentiating bacterial infection from others and starting empiric treatment.

Keywords:

16S rRNA, body fluids, ribosomal, sequence analysis

Introduction

Several rapid methods for disease detection have evolved over time. Delay in identification of the causative agent is a diagnostic dilemma faced in many life-threatening infections. Conventional diagnosis of bacterial infection is time-consuming as it has to go through several stages including culture and phenotypic identification requiring minimum of 24 h of incubation.[1] Sometimes, the disease remains undetected due to slow-growing organisms or non-cultivable nature of the organism. Rapid detection of pathogens in clinical samples will help accurate treatment.[2] Molecular methods of detection are proving to bridge some of these gaps.[3]

Targeting conserved regions of microbial genomes, particularly the 16S rRNA of

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bacteria by polymerase chain reaction (PCR), combined with sequence analysis of the products is a novel approach to rapid detection of pathogens from clinical samples. 16S rRNA is a broad-range bacterial genome which is targeted directly from the clinical specimen. Subsequent sequencing of the PCR product and using nucleotide Basic Local Alignment Search Tool (BLAST) to compare with the data available in the gene bank has increased the spectrum for identification of pathogens. This technology has been used in the diagnosis of sepsis, endocarditis, meningitis, arthritis, pleural effusion and bone and joint infections.[4-9] The protocol for 16S rRNA gene PCR in routine diagnostic workflow is rapid and simple.[1]

We aimed to evaluate the use of 16S rRNA PCR for detecting bacterial pathogens in infected body fluids and compare with conventional culture method.

Materials and Methods

The study was cleared by the Institute Ethics Committee (IEC-RC/14/110) and a waiver of consent was obtained as the study did not involve human participants and the tests were performed on samples received in the laboratory for conventional culture. This was a cross-sectional study conducted between January and December 2015 in the Department of Microbiology. A convenient sampling of 100 consecutive body fluids sent for bacterial culture was included. Samples tested included synovial fluid, cerebrospinal fluid (CSF), ascitic fluid and pleural fluid.

These were aseptically aliquoted into two fractions, one each for culture and 16S rRNA. Conventional culture was done as per standard microbiological protocols.[10] All samples were stored at −20°C for 16S rRNA PCR. Following centrifugation of the second aliquot, DNA was extracted using an extraction kit, QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions.

Amplification of 5 µl of the extracted DNA along with positive control (*Escherichia coli* ATCC 25922) and negative control (distilled water) was done with 0.5-mM specific primers as mentioned in Table 1 adapted from Woo *et al*.,[11] 200 mM of each dNTP, 10 mM KCl, PCR buffer, 1.5 mM $MgCl_2$ and 1.0 U Taq polymerase (Ampliqon). Amplification conditions for PCRs were as follows: Five minutes at 94°C to denature the DNA, followed by 40 cycles of denaturation at 94°C for one minute, primer annealing at 62°C for one minute and strand extension at 72°C for two minutes on an Eppendorf thermal cycler. PCR products were subjected to gel electrophoresis on a 1.5% agarose gel with ethidium bromide, and DNA bands were visualised.

This procedure took around four hours. Those samples which were found to be positive for 16S rRNA at 1343-bp band were further sequenced (Eurofins Private Limited, Bengaluru, Karnataka, India). Purified amplicons were sequenced in Applied Biosystems in the forward and reverse direction in separate reactions and in duplicate. Organisms were identified by nucleotide BLAST comparing sequences to a database library of known 16S rRNA gene sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) by multiple sequence alignment. The bacterial source of the sequence was identified by matching it with a sequence with the highest maximum identity score from the GenBank database. Results of the molecular method were compared with the conventional culture.

Statistical analysis

Comparison of 16S rRNA PCR and culture for detection of pathogens was performed using the McNemar's test for significance. $P < 0.05$ (two-tailed) was considered significant. Sensitivity and specificity of culture and 16S rRNA were determined using IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

Results

Samples positive by culture were 3 (3%), while bacterial 16S rRNA was amplified in 13 of the 100 (13%) samples tested by the 1343-bp PCR protocol [Table 2]. Maximum score, total score and *E* value and percentage of identification for each of the positive cases by PCR are described in Table 3.

Concordance of culture and polymerase chain reaction results

Ninety samples yielded concordant results in culture and PCR. Three samples had identical positive results such as *E. coli* from ascitic fluid, *Streptococcus pneumoniae* from CSF and *Staphylococcus aureus* from pleural fluid. Eighty-seven samples were negative by both culture and PCR methods. A concordance of PCR and culture for both identical positive and negative samples was (3 + 87)/100, i.e. 90%.

Table 1: Details of primer sequences used in this study

Fragment	Primer	Sequence	Nucleotide position	Reference
1343 bp	LPW57	5'-AGTTTGATCCTGGCTCAG-3'	10-27	Woo *et al*., 2001
	LPW58	5'-AGGCCCGGGAACGTATTCAC-3'	1370-1389	

Polymerase chain reaction-positive and culture-negative results

PCR was positive in 13 samples. Uncultured bacterial clones were identified in four ascitic fluid and one CSF sample; enterobacter species were found in one ascitic fluid sample. One of the CSF samples had lactobacillus spp. [Table 4]. Three samples (2 – CSF and 1 – ascitic fluid) were positive for 16S rRNA but could not be sequenced.

None of the samples were positive by culture but negative by PCR. The detection rate of 16S rRNA PCR (13%) was higher than that of culture (3%) ($P = 0.0009$). Compared to culture, the diagnostic sensitivity and specificity of the PCR were 100% and 89.7%, respectively.

Discussion

Culture is a definitive diagnostic criterion often referred to as the gold standard. However, in practice, it is often inconclusive due to several factors, low bacterial load, prior antibiotic treatment and fastidious or non-cultivable nature of the organism. Molecular detection methods have overcome some of these lacunae. Detection of a pan-bacterial genome component, the 16S rRNA by PCR has increased the detection of bacterial pathogen. In the present study on body fluids, the 16S rRNA was detected in 13% of the specimens compared to 3% by culture. This was comparable to Grif *et al*., who found that among 47 synovial fluid specimens, 3 (6.4%) were positive by culture and 9 (19.1%) were positive by PCR.[3] Similar results have also been reported by Akram *et al*.,[12] suggesting that PCR assay is useful for the early diagnosis of bacterial infections. Several factors could contribute to the superiority of a molecular detection method over conventional culture. Besides factors mentioned earlier, compromised viability of fastidious organisms during transport and quality of media and culture conditions[13,14] may also be contributing factors.

Table 2: Various body fluids tested and number of samples positive for 16S rRNA polymerase chain reaction

Body fluid	Total samples tested	Number positive for culture	Number positive for 16S rRNA
Ascitic fluid	55	1	8
CSF	26	1	4
Pleural fluid	16	1	1
Synovial fluid	3	0	0
Total	100	3	13

CSF: Cerebrospinal fluid

The specificity of PCR compared to culture was 89.7%, similar to other studies.[3,12]

Mere detection of the 16S rRNA may be helpful in narrowing down the aetiology to bacterial and non-bacterial which may help in initiating empiric therapy. As a logical follow-up, sequencing of the PCR product must be done, it plays a vital role in the accurate identification of bacterial isolates. This will help in targeting specific antibiotic therapy.

However, the uncultured bacterial clone proved difficult to interpret due to their unique genome which did not match the known genomes in the data bank. From clinical perspective, this could pose a challenge in initiating targeted empiric therapy Literature shows several instances of uncultured bacterial clones found in the environment. Human isolates have still remained unidentified.

Lactobacillus spp. causing meningitis, a rare case, could be a pathogen.[15]

A major limitation of the molecular technique is the absence of an antibiotic susceptibility testing, unless resistance genes are detected simultaneously. This is offset by the rapidity of establishing an aetiological diagnosis. Newer automated culture methods with rapid identification systems including antibiotic susceptibility testing have improved the turnaround time for diagnosis. Detection of 16S rRNA is an improvement on decreasing the time from collection to report. However, despite encouraging results, 16S rRNA detection and sequencing in clinical microbiology laboratories requires improved

Table 3: Sequencing results of samples positive for 16S rRNA polymerase chain reaction

Sample number	Maximum score	Total score	E	Percentage of identification	Organism
3	1874	1874	0.0	97	*E. coli*
6	1779	1779	0.0	97	Uncultured bacterium clone
18	283	283	1e-71	83	Uncultured bacterium clone
19	161	161	3e-35	90	Uncultured bacterium clone
26	676	676	0.0	87	Uncultured bacterium clone
33	132	132	3e-26	86	Enterobacter species
39	1550	6196	0.0	97	*S. pneumoniae*
55	311	311	5e-80	84	Uncultured bacterium clone
56	1953	1953	0.0	97	Lactobacillus species
59	1336	1336	0.0	93	*S. aureus*

S. aureus: Staphylococcus aureus; S. pneumonia: Streptococcus pneumoniae; E. coli: Escherichia coli

Table 4: Comparison of culture and 16S rRNA polymerase chain reaction assay

Sample number	Body fluid	Culture growth	Sequencing result
3	Ascitic fluid	*E. coli*	*E. coli*
6	Ascitic fluid	No growth	Uncultured bacterium clone
18	Ascitic fluid	No growth	Uncultured bacterium clone
19	Ascitic fluid	No growth	Uncultured bacterium clone
26	Ascitic fluid	No growth	Uncultured bacterium clone
33	Ascitic fluid	No growth	Enterobacter species
39	CSF	*S. pneumonia*	*S. pneumonia*
55	CSF	No growth	Uncultured bacterium clone
56	CSF	No growth	Lactobacillus species
59	Pleural fluid	*S. aureus*	*S. aureus*

CSF: *Cerebrospinal fluid; S. aureus: Staphylococcus aureus; S. pneumonia: Streptococcus pneumonia; E. coli: Escherichia coli*

guidelines for interpretation of results. Another major drawback is the need for additional/supplementary methods for bacterial species which cannot be identified confidently by 16S rRNA sequencing alone.[9] The cost of molecular tests has come down with increasing use of these tests in diagnostic laboratories, yet they are much higher than conventional culture. Even as our results were promising, we have not done a cost evaluation to assess its utility as a routine test, which is a limitation of the present study. Further studies are needed to improve its sensitivity and evaluate its feasibility and cost-effectiveness.

Conclusions

The 16S rRNA gene sequencing appears to be a novel tool for diagnosing infections from body fluids. The major advantage over conventional culture is its ability to detect and identify cultivable, non-cultivable and non-viable bacteria. Even though 16S rRNA detection in body fluids appears promising, it needs to be further evaluated before it can be widely used in clinical diagnostic laboratory.

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Conflicts of interest
There are no conflicts of interest.

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