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Identification of Bacteria in Formalin-Fixed, Paraffin-Embedded Heart Valve Tissue via 16S rRNA Gene Nucleotide Sequencing

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We applied 16S rRNA gene sequencing to identify bacterial species present in formalin-fixed, paraffin-embedded heart valve tissue. In 40% (12/30) of the cases, we were able to identify the bacterium to the species-genus level. For more recent cases (≤4 years), the success rate was significantly improved, to 70% ($P < 0.001$).

Infective endocarditis (IE) is usually diagnosed by clinical, histological, and/or microbiological parameters according to the Duke scheme (5). Blood culture is the most frequently used method of identifying bacterial etiologic agents of IE. For patients undergoing heart valve replacement surgery, direct heart valve tissue cultures may also be used to identify the bacterial agent. Approximately 2.5 to 31% of IE cases are culture negative due to noncultivatable organisms or previous antimicrobial treatment (1, 2, 10). Without etiological identification, choosing an effective therapeutic regiment for IE can be challenging. For fresh/frozen heart valve tissue, the application of broad-range PCR for amplification of bacterial 16S rRNA genes for sequencing has significantly improved the identification of bacterial agents in untreated culture-negative IE and antimicrobially treated cases (6, 8, 9, 13).

In clinical practice, one encounters situations where the diagnosis of IE is made based on clinical/histological evidence, as blood cultures are negative and the surgically removed heart valve tissue is formalin fixed and paraffin embedded (FFPE). When the diagnosis is based solely on histological findings, bacterial species identification has historically been limited to Gram staining of tissue sections. 16S rRNA gene sequencing is a viable option for bacterial identification in this situation. To the best of our knowledge, there are no published data systematically addressing the effectiveness of the 16S rRNA gene sequencing method for bacterial identification in FFPE heart valve tissue. In a recent review article of diagnostic methods for infective endocarditis, it was simply stated that PCR and sequencing approaches "are difficult to perform on paraffin sections" (11). To address this clinical/technical dilemma, we conducted a retrospective study to evaluate a procedure using 16S rRNA gene sequencing to identify bacterial species present in FFPE heart valve tissue.

This study, approved by the Institutional Review Board of Hackensack University Medical Center, includes cases diagnosed as IE in the Department of Pathology, Hackensack University Medical Center. Paraffin blocks were used for DNA extraction. Patient charts were reviewed by members of the infectious disease team to confirm the clinical diagnosis of IE and the blood culture results. An archival search from the files of the Department of Pathology was performed, covering a 10-year period (1995–2004). The slides and available paraffin blocks were retrieved for all cases diagnosed as endocarditis based on histopathologic evidence, and only those cases with confirmed bacterial colonies identified on tissue sections by hematoxylin and eosin and/or Gram staining were selected for this study. The slides and corresponding paraffin blocks were marked to localize the bacterial colonies for DNA extraction, which was carried out in a class II biological safety cabinet in an area designated exclusively for DNA extraction. DNA extraction was performed using a Puregene DNA isolation kit from Gentra (Gentra Systems, Minneapolis, MN) following the manufacturer's instructions. Primers 0008F (5′-AGAGTT TGATCCTGGCTCAG-3′) and 0532R (5′-TACCGCGGCTG CTGGCAC-3′) (7) were used to amplify the first 500 bp of the 16S rRNA gene. The annealing temperature was 59°C. BigDye Terminator v3.1 cycle sequencing kits and an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) were used for DNA sequence determination. The 16S rRNA gene sequences obtained were compared with those available in the Microseq 500 library and the GenBank databases (http://www.ncbi.nlm.nih.gov/BLAST/). A match of ≥99% with either a Microseq 500 or GenBank sequence was considered identification to the species level; ≥97% matching was considered identification to the genus level (4).

A total of 30 paraffin blocks were analyzed by broad-range 16S rRNA gene PCR and nucleotide sequencing. In 40% (12/30) of the cases, we were able to identify the bacteria to the species and/or genus level. Among the positive cases, 9/12 (75%) were positive by both culture and PCR/sequencing, and 3/12 (25%) were positive by PCR/sequencing only (Table 1). The success rate for sequence analysis was 100% (12/12).

The bacterial identification rate for newer paraffin tissue sections (i.e., tissues collected after 2001) was 70%, which proved to be significantly higher ($P < 0.001$) than that for older tissue (i.e., tissue collected during 1995–2000), which was only 18%.

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TABLE 1. Correlation of bacterial identification by 16S rRNA gene PCR/sequencing and blood or heart valve tissue culture

Case no. (yr of sample)	Organism identified by:	
	16S rRNA gene PCR/sequencing	Blood or heart valve tissue culture
1 (2001)	*Streptococcus bovis*	*Streptococcus bovis*
2 (2003)	*Enterococcus faecalis*	*Streptococcus agalactiae*
3 (2002)	*Staphylococcus aureus*	*Staphylococcus aureus*/CoNS
4 (2001)	*Enterococcus faecalis*	*Enterococcus faecalis*/*CoNS*
5 (2002)	*Staphylococcus lugdunensis*	CoNS
6 (2001)	*Staphylococcus aureus*	*Staphylococcus aureus*
7 (2002)	*Staphylococcus aureus*	Negative[a]
8 (2002)	*Streptococcus* species	Negative[a]
9 (2003)	*Gemella haemolysans*	CoNS
10 (2000)	*Gemella haemolysans*	Negative[a]
11 (1995)	*Staphylococcus epidermidis*	CoNS
12 (1996)	*Enterococcus faecalis*	*Enterococcus faecalis*

[a] Gram staining showed gram-positive cocci.

The correlation between 16S rRNA gene sequencing and blood/heart valve culture was 75% (9/12) (if the blood/tissue culture was negative, then the organism identified by PCR/sequencing that correlated with tissue Gram stain morphology was considered to correlate). After further analysis, we concluded that in two cases (cases 5 and 9, Table 1), 16S rRNA gene sequencing gave a more precise identification. The adjusted correlation rate was 92% (11/12).

The major benefit of organism identification in heart valve tissues from patients with IE is to guide clinicians towards the selection of appropriate antimicrobial therapy. In our study, PCR/sequencing identified bacterial species in about 40% (12/30) of paraffin block samples analyzed. Since this was a retrospective study, the ages of the resected heart valve tissue samples ranged from a few days to 10 years. In our experience, bacterial DNA isolation/PCR amplification was more successful in tissue samples stocked for <4 years (70% positive rate), while the success rate was much lower (18%) for samples collected >4 years ago. In day-to-day clinical situations, when a patient's heart valve is surgically removed, it is more likely be recently formalin fixed and paraffin embedded, and we expect the success rate of the PCR/sequencing approach to be similar to that obtained using more recent tissue blocks.

We do not know the mechanism for why 16S rRNA gene sequencing for bacterial identification from older paraffin blocks was more difficult than that from more recent blocks; we speculate that the longer the formalin-fixed tissue stays in paraffin, the more cross-linking of histone-like proteins to DNA or fragmentation/degradation of genomic DNA occurs over time.

Note the significance of identifying *Staphylococcus lugdunensis*, as shown in case 5 (Table 1), from FFPE heart valve tissue. *S. lugdunensis* has been reported to be a very aggressive pathogen for IE relative to other coagulase-negative staphylococci (CoNS) and is associated with poorer clinical outcomes (3, 14). In blood culture, the isolate was simply identified as CoNS. Misidentification of *S. lugdunensis* may cause serious consequences in the situation of IE.

With regard to the identification of *Gemella* species versus CoNS (case 9, Table 1), we postulate that CoNS was misidentified by performing only the coagulase test on gram-positive cocci in clusters and not performing a catalase test, therefore causing misidentification. Another significant discrepancy was the case where *Streptococcus agalactiae* was identified by culture, whereas PCR/sequencing from the FFPE heart valve tissue identified *Enterococcus faecalis* (case 2, Table 1). From chart review, the patient was initially treated with ceftriaxone and later switched to ampicillin, gentamicin, and vancomycin. The treatment covered both *Streptococcus agalactiae* and *Enterococcus faecalis*. We could not rule out the possibility that the identification of *E. faecalis* was due to contamination of the specimen.

In cases 3 and 4, both blood cultures grew two isolates, while 16S rRNA gene sequencing only identified one isolate in each case. It is interesting that in both cases, CoNS was the second isolate from blood culture, which probably represented contamination by a skin organism during blood drawing (15), while 16S rRNA gene sequencing identified the real pathogens, *Staphylococcus aureus* (case 3) and *E. faecalis* (case 4). The 16S rRNA gene sequencing method has the advantage of directly identifying the bacterial pathogen from the location of the disease, i.e., the heart valve. Polymicrobial IE occurs at a low frequency (1 to 2%) (12). A limitation of 16S rRNA gene sequencing methods is that they may identify only one organism or a mixed sequence amplified by PCR, and direct nucleotide sequencing is not possible in this situation.

Since the heart valve tissues were not processed under sterile conditions during formalin fixation and paraffin embedding, good clinical/microbiological judgment should be practiced in analyzing the bacterial identification results. Bacteria identified by this approach should be interpreted in correlation with the Gram stain bacterial morphology of the original tissue section.

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