Utility of Histologic and Histochemical Screening for 16S Ribosomal RNA Gene Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue for Bacterial Endocarditis

Isaac H. Solomon, MD, PhD, Chieyu Lin, MD, PhD, Katharine L. Horback, MD, Sanjat Kanjilal, MD, MPH, Vanesa Rojas-Rudilla, MS, Manfred Brigl, MD, Alvaro C. Laga, MD, MMSc, Neal I. Lindeman, MD, and Robert F. Padera Jr, MD, PhD

From the Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA.

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ABSTRACT

Objectives: *16S ribosomal RNA (rRNA) sequencing is a powerful but expensive tool for the identification of bacteria in culture-negative endocarditis. Histologic criteria to screen formalin-fixed, paraffin-embedded (FFPE) specimens for testing are evaluated.*

Methods: *Sixty-eight cases of infective endocarditis and controls were histologically reviewed and analyzed by 16S rRNA gene sequencing.*

Results: *Sequencing identified a specific pathogenic organism in 33 (49%) of 68 cases with acute inflammation and in 0 of 10 controls (*P = *.004). Visualization of organisms by Gram or Grocott methenamine silver stains had the strongest association with positive sequencing, while antibiotic treatment effect and acid decalcification decreased sensitivity. Molecular identifications were concordant with blood culture results in 90% of the cases, and a positive sequencing result was obtained in approximately half of the cases with negative valve cultures.*

Conclusions: *Histologic screening criteria are extremely helpful for identifying cases likely to be positive by molecular testing and can provide significant cost savings in filtering out low-yield specimens.*

Infective endocarditis may show minimal to severe endocardial inflammation due to infection on the surface of native and prosthetic heart valves and occurs in three to 10 per 100,000 individuals.[1] Patients with infective endocarditis have significant morbidity due to sepsis and embolization, resulting in death in 10% to 26% of cases.[2] A variety of causative organisms have been identified, most commonly Gram-positive cocci, including *Staphylococcus* spp, *Streptococcus* spp, and *Enterococcus* spp.[3] Additional pathogens include Gram-negative bacilli such as the HACEK group (*Haemophilus*, *Aggregatibacter*, *Cardiobacterium*, *Eikenella*, *Kingella* species) and, less commonly, *Coxiella burnetii*, *Tropheryma whipplei*, *Bartonella* spp, *Brucella* spp, *Mycobacterium* spp, and fungi such as *Candida* spp or *Aspergillus* spp. Treatment of infective endocarditis consists of administration of a prolonged course of intravenous antiinfectives, with surgical resection of the valve reserved for severe cases.[1] Appropriate antibiotic selection relies on the identification of a specific causative organism; however, up to 71% of blood cultures and 94% of valve cultures have been reported negative in some studies due to prior antibiotic treatment or involvement by an unculturable organism.[2] Occasionally, tissue is not submitted for culture at the time of surgery due to lack of clinical suspicion perioperatively.[4] Serologic testing (eg, *C burnetii* and *Bartonella* spp) and directed polymerase chain reaction (PCR) (eg, *C burnetii*, *T whipplei*, and *Bartonella* spp) are available for some organisms but must

be individually selected and can be expensive.[3] Similarly, targeted syndromic panels may provide increased sensitivity compared with broad-range testing[5] but will not detect rare or unusual organisms. Sequencing the bacterial ribosome 16S ribosomal RNA (rRNA) gene has provided a powerful tool for species-level identification of bacteria from culture isolates and from a variety of primary tissue specimens.[6,7] 16S rRNA sequencing of fresh or frozen heart valve tissue has been shown to have increased sensitivity (33%-92%) compared with valve culture (8%-44%).[8-12] Formalin-fixed, paraffin-embedded (FFPE) tissue has also been effectively used for 16S rRNA sequencing,[13-16] but there are no consensus histologic guidelines to select appropriate cases, and there are currently no molecular results incorporated into the Duke criteria for diagnosis of infective endocarditis.[17,18] Thus, the goal of this study was to identify histologic features associated with positive 16S rRNA gene sequencing in bacterial endocarditis FFPE specimens to maximize the diagnostic yield and minimize the expenditure of resources on samples unlikely to provide clinically useful information.

Materials and Methods

This study was approved by the Institutional Review Board of the Brigham and Women's Hospital (Boston, MA). Heart valve resection specimens with Gram and Grocott methenamine silver (GMS) stains to evaluate for endocarditis were identified from a 5-year period from 2012 to 2017. Electronic medical records were reviewed, including blood and valve culture results and prior molecular testing, to determine if a causal agent had been identified. A representative subset of cases (78/142) was selected for further evaluation, including 68 cases with active endocarditis and 10 cases lacking acute inflammation, encompassing a variety of organisms, both prosthetic and native heart valves, as well as tissue treated with acid for decalcification prior to processing and embedding. Histologic review was performed independently by three pathologists with subspecialty expertise in infectious disease (I.H.S. and A.C.L.) or cardiovascular pathology (R.F.P.) and included H&E, Gram, and GMS stains for all cases. In addition, Warthin-Starry (WS) stains were performed for all cases with negative Gram and GMS stains.

Molecular identification was performed by the Center for Advanced Molecular Diagnostics at the Brigham and Women's Hospital, based on 16S rRNA V1/V2 gene sequencing methods developed by the University of Washington.[19,20] Genomic DNA was isolated from three 20-µm scrolls using the QiAMP DNA mini kit (Qiagen), and PCR amplification was performed using primers 16S_V1_7F (AGAGTTTGATCNTGGCTCAG) and 16S_V2_338R (CTGCTGCCNCCCGTAGGAG) with KAPA2G HotStart ReadyMix (Sigma-Aldrich). PCR reactions were examined on a 2% agarose gel, and products were directly sequenced or isolated with the QIAquick Gel Extraction kit (Qiagen) if multiple bands were visualized. Sequencing was performed with the amplification primers, analyzed with an ABI 3130xl Genetic Analyzer (Applied Biosystems), and processed with Sequencing Analysis 5.2 (Applied Biosystems). ABI trace files were then analyzed with CodonCode Aligner (CodonCode Corporation), with forward and reverse reads trimmed to meet quality criteria and assembled into a single contig. Contigs were fed into the 16S RipSeq Single database (Pathogenomix) or nucleotide blast with 16S rRNA sequences (Bacteria and Archaea) database for identification to genus or species level per guidelines established by the Clinical and Laboratory Standards Institute.[21] Microbial DNA from organisms recurrently identified in histology laboratory reagents, including *Geobacillus* spp, *Meiothermus silvanus*, *Acinetobacter radioresistens*, and *Sphingomonas* sp, was classified as contamination rather than pathogenic and excluded from further analysis.

Statistical significance was determined by Fisher exact test, with a P value of less than .05 considered significant.

Results

A total of 68 cases of active endocarditis and 10 negative controls were evaluated by 16S rRNA gene sequencing for identification of a causative pathogen (**Table 1**, groups 1-4). Active endocarditis cases were characterized by a neutrophilic infiltrate with variably sized vegetations and often contained abundant bacteria **Image 1**. Overall, 33 (49%) of 68 cases with active endocarditis and zero (0%) of 10 non-inflammatory controls were positive for a likely pathogen (P = .004) by 16S sequencing. In addition, microbial DNA was detected in 25 (37%) of 68 active endocarditis cases and eight (80%) of 10 controls, corresponding to *Geobacillus* spp (n = 13), *M silvanus* (n = 11), *A radioresistens* (n = 8), and *Sphingomonas* sp (n = 1). These thermophilic organisms were first isolated in industrial settings, have not been associated with human disease, and can be detected in histology laboratory reagents; these were therefore classified as contaminants in this study and excluded from further analysis.[22,23]

Gram stains highlighted Gram-positive cocci in 41 cases singly, in chains, or in clusters, as well as Gram-positive bacilli in two cases. 16S sequencing was positive

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Table 1
16S Ribosomal RNA Sequencing Results by Histologic Findings

	Histologic Findings			16S Positive, No. (%)				
					Decalcification		Antibiotic Treatment Effect	
Group No.	Gram	GMS	AI	Total Positive Cases	Yes	No	Yes	No
1	+	+	+	28/43 (65)	4/11 (36)	24/32 (75)	5/13 (38)	23/30 (77)
2	–	+	+	5/15 (33)[a]	0/2 (0)	5/13 (38)	2/12 (17)	3/3 (100)
3	–	–	+	0/10 (0)	0/2 (0)	0/8 (0)	—	—
4	–	–	–	0/10 (0)	0/3 (0)	0/7 (0)	—	—

AI, acute inflammation; GMS, Grocott methenamine silver; +, positive; –, negative.
[a]Includes three cases of Gram-negative bacilli (*Cardiobacterium hominis*, *Haemophilus parainfluenzae*, and *Streptobacillus moniliformis*).

in 28 (65%) of 43 cases with positive Gram stains (group 1). GMS stains were positive in all 43 cases with positive Gram stains, as well as 15 additional cases (13 cocci and two bacilli). A likely pathogenic organism was identified by sequencing in 33 (57%) of 58 GMS-positive cases overall and in five (33%) of 15 cases positive for GMS but negative by Gram stain (group 2). Cases with negative Gram and GMS stains were also negative by WS stain, regardless of whether acute inflammation was present (n = 10; group 3) or absent (n = 10; group 4). 16S sequencing was negative for all cases in groups 3 and 4.

Endocarditis valve resections in this study included 52 native heart valves (24 aortic, 25 mitral, and three tricuspid) and 16 prosthetic valves (12 aortic and four mitral). There was no difference in the rate of 16S sequencing positivity between native and prosthetic valves (24/52 [46%] vs 9/16 [56%]; $P = .6$). Decalcification by acid treatment prior to tissue processing and embedding was performed in 15 active endocarditis cases and three negative controls. 16S sequencing was positive in four (36%) of 11 Gram-positive (group 1) cases that underwent decalcification and in 24 (75%) of 32 cases that did not ($P = .03$). In cases positive by GMS stain only (group 2), 16S sequencing was positive in zero (0%) of two cases that underwent decalcification and in five (38%) of 13 cases that did not ($P = .5$). Antibiotic treatment effect, characterized by loss of Gram positivity and increased size of organisms on GMS stains, was evident in 13 Gram-positive (group 1) cases and 12 GMS-only (group 2) cases. Sequencing was positive in five (38%) of 13 group 1 cases with treatment effect compared with 23 (77%) of 30 ($P = .03$) without, as well as in two (17%) of 12 group 2 cases with treatment effect and three (100%) of three without ($P = .02$).

Of the 68 cases of active endocarditis, 63 were previously associated with a pathogen identified by blood or valve culture, and an additional two cases were positive by reference laboratory 16S rRNA gene sequencing at an outside institution. A pathogen was identified by sequencing in this study in 33 (49%) 68 cases, including 19 *Streptococcus* spp, six *Staphylococcus* spp, four *Enterococcus faecalis*, and one each of *Aerococcus urinae*, *Cardiobacterium hominis*, *Haemophilus parainfluenzae*, and *Streptobacillus moniliformis*. Sequencing results were completely concordant or more specific than the identification made by standard microbiology laboratory workup in 28 (90%) of 31 cases and were concordant with both cases with reference laboratory 16S rRNA sequencing (*S moniliformis* and *Streptococcus* sp). A pathogen was identified in 16 (47%) of 34 cases with negative valve cultures and in four (44%) of nine cases with negative blood cultures. Identifications made by sequencing were concordant with valve culture results in 12 (100%) of 12 cases and with blood cultures in 26 (90%) of 29 cases. Two discrepant cases differed at the species level (*Streptococcus gordonii* vs *Streptococcus anginosis* and *Streptococcus sanguinis* vs *Streptococcus oligofermentans*), while the third was identified as *Staphylococcus lugdunensis* by blood culture and *Streptococcus* spp by sequencing. In addition, two cases without a previous identification were positive by sequencing (*Streptococcus gallolyticus* and *Streptococcus dysgalactiae*).

Discussion

The results of this study strongly support the utility of histologic and histochemical screening of infectious endocarditis FFPE specimens for 16S rRNA gene sequencing. A plausible causative organism was identified in nearly half of the cases with acute inflammation (49%) and nearly half of the cases with negative valve cultures (47%). The overall sensitivity and specificity were 44% and 100%, respectively, with high concordance (28/31, 90%) between the original diagnosis obtained through traditional microbiologic diagnostics and the 16S sequencing results. In two of the three discrepant cases, there was agreement at the genus but not species level (*S gordonii* vs *S anginosis* and *S sanguinis* vs *S oligofermentans*). In both cases, the original diagnosis was based on blood culture results from a referring institution, and no further information was

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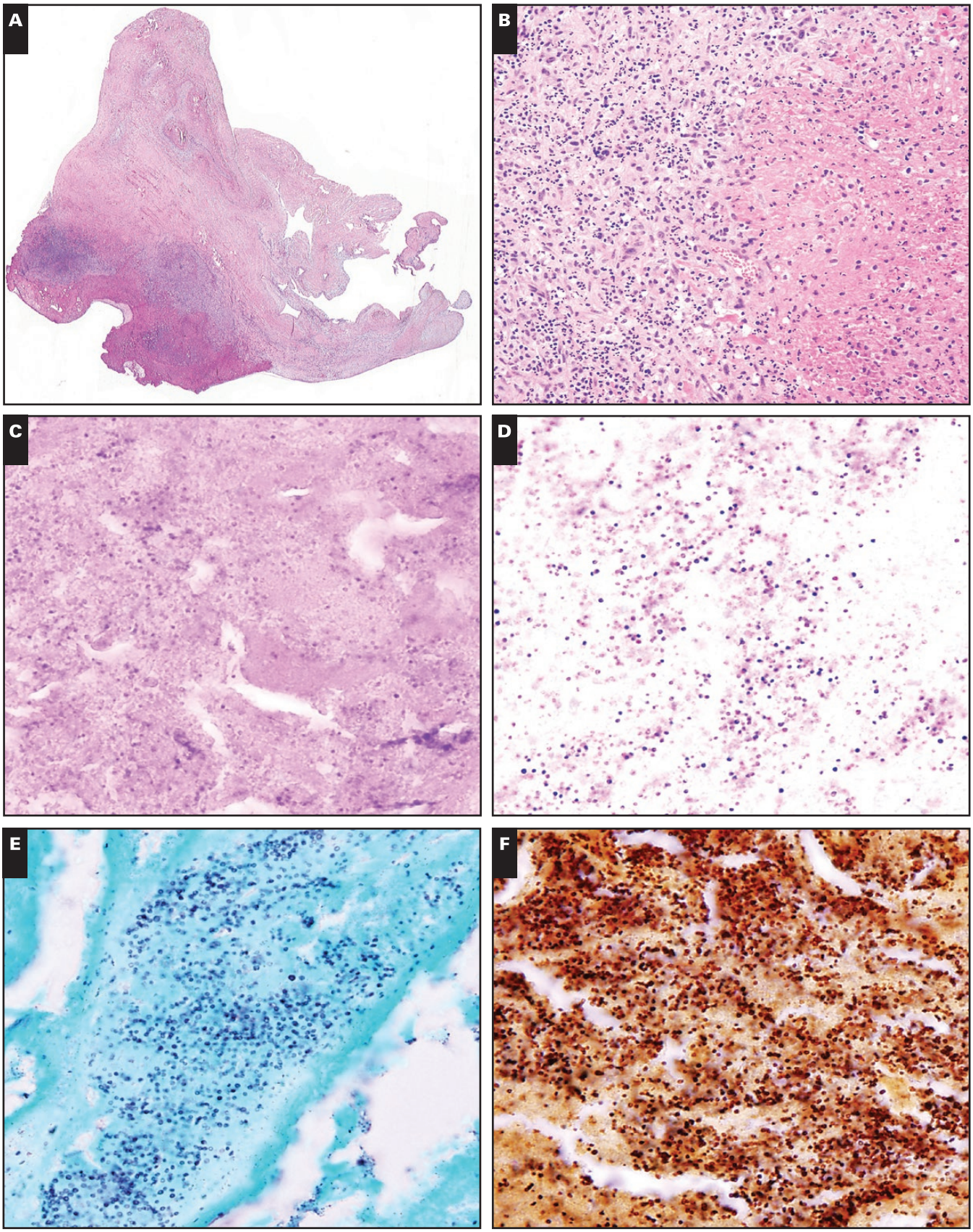

Image 1 Histologic findings diagnostic of infective endocarditis. **A**, Low-power view of a resected tricuspid valve leaflet shows a large vegetation (H&E). **B**, A higher power image from the vegetation shows dense acute inflammation and fibrin deposition (H&E). Cocci are visible on H&E (**C**), Gram (**D**), Grocott methenamine silver (**E**), and Warthin-Starry stains (**F**). Images taken with slide scanner (**A**) or at ×40 (**B-F**).

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available regarding the specific testing method, raising the possibility of a biochemical/phenotypic misidentification. The third discrepant case had *S lugdunensis* identified by blood culture and *Streptococcus* spp (most consistent with *Streptococcus mitis* or *Streptococcus pneumoniae*) identified by sequencing. On detailed chart review, the patient was determined to have a concomitant soft tissue abscess along with infective endocarditis, making it possible that there were separate sources leading to seeding of the bloodstream.

The presence of bacteria on Gram (65%) or GMS (57%) was strongly associated with a positive sequencing result. These results are similar to a prior study with FFPE endocarditis tissue in which 12 (40%) of 30 cases with observable bacteria were positive by sequencing.[15] WS stains were performed only for cases with negative Gram and GMS stains but likely would have exhibited a similar correlation if available for all cases. In the cases with observable organisms (groups 1 and 2), sequencing yield was markedly decreased by acid decalcification (4/13 [31%], $P = .054$) or by extensive antibiotic treatment effect (7/25 [28%], $P = .0002$), such that 24 (86%) of 28 cases without decalcification or antibiotic treatment effect were positive by 16S sequencing, and only four (16%) of 25 cases negative by sequencing lacked either acid decalcification or antibiotic treatment effect ($P < .0001$). To determine how many organisms would be necessary for a positive molecular identification, a semiquantitative scale of zero (0), rare (1+), and abundant (2+) organisms was proposed. However, only three cases fitting in the rare (1+) category were identified in this cohort, two of which were positive by 16S sequencing, while the third had been treated with acid decalcification. Based on these limited results, there was no clear minimum number of organisms to predict a positive 16S sequencing result, and other factors, including extent of antibiotic treatment prior to surgery and processing of the tissue specimen prior to sequencing, appear to have a stronger effect on sequencing results.

Despite the exclusion of cases lacking observable bacteria in the largest previously published study of endocarditis from FFPE,[15] it was unexpected that all histologically negative cases in this study would be negative by sequencing. FFPE case series with a variety of specimen types, including endocarditis samples, have demonstrated positive molecular testing without visible bacteria.[16] The persistence of bacterial DNA after clearance by antibiotics, a major argument against incorporation of sequencing results into the Duke criteria, has been well documented.[24] While one of the major advantages of testing FFPE tissue vs fresh or frozen tissue is the ability to screen for specimens that have visible organisms to increase molecular diagnostics yield, formalin fixation has the opposite effect by reducing the quantity and length of DNA isolated, limiting the overall quality of sequencing data.[25] The use of FFPE tissue includes many additional sources for environmental contamination to be introduced in the nonsterile gross room and histology laboratory, which may drown out the signal of pathogenic organisms. Nonpathogenic environmental bacteria, including *M silvanus*, *A radioresistens*, *Geobacillus jurassicus*, and *Sphingomonas hankookensis*, were identified in a subset of cases from all sample groups in this study.[22,23] Contaminants were also found in laboratory reagents, including freshly opened bags of paraffin, highlighting the need for individual laboratories to track recurrent environmental organisms, as well as the need for histologic correlation when possible. Utilization of next-generation sequencing could increase sensitivity compared with the Sanger sequencing employed in this study but may also increase the false-positive rate and decrease specificity unless strict quality control measures are employed.[26] Geographic location and specific comorbidities may also explain the differences in endocarditis-associated pathogens between study cohorts, thus affecting the report rates of sequencing positivity.[27,28] While some bias was introduced in the selection of active endocarditis cases to ensure a variety of pathogens, all cases lacking observable organisms during the study period were included (n = 10) to maximize the chances of identifying a positive case by sequencing.

Based on this study and the published literature, molecular testing of FFPE tissue can produce clinically useful data in a high percentage of infectious endocarditis cases when histologic criteria are used for screening. We recommend routine use of Gram and GMS staining for all surgical pathology specimens in cases with acute inflammation or clinical suspicion for infectious endocarditis, with WS reserved as a second-line test or first-line test if a Gram-negative organism is high on the differential. Based on our results, cases with negative cultures and serology should be referred for 16S rRNA gene sequencing if acute inflammation is present. This study included only a small number of non-Gram-positive cocci and no cases of Q fever, bartonellosis, brucellosis, or Whipple disease; additional studies are required to confirm the results for these organisms or to suggest individual pathogen or syndromic panel targeted testing.[5] As suggested by Liesman et al,[3] cases negative for 16S rRNA testing or with mild or minimal inflammation should be considered for *C burnetti*, *Bartonella* spp, and *T whipplei* PCR, while cases exhibiting macrophage-predominant chronic inflammation should be stained with periodic acid–Schiff with diastase and considered for *T whipplei* PCR prior to 16S sequencing. FFPE

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blocks with the highest number of organisms should be selected for testing, and alternative samples should be used whenever possible if the specimen has been extensively decalcified. These recommendations may also be useful for guiding molecular testing of other FFPE tissue specimens; however, sensitivity and specificity are likely to vary depending on additional factors. Sterility of the tissue site and the likely pathogens involved, as well as how distinguishable they are based on partial 16S rRNA gene sequencing, may require a higher or lower threshold of histologic features for screening. While the main purpose of this study was to provide guidance for molecular testing of FFPE tissue, histologic screening can also be used to inform testing of fresh/frozen tissue, and it is recommended that fresh samples be sterilely aliquoted for potential molecular studies prior to histologic processing.

Corresponding author: Robert F. Padera Jr, MD, PhD; rpadera@bwh.harvard.edu.

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