Acid-fast Smear and Histopathology Results Provide Guidance for the Appropriate Use of Broad-Range Polymerase Chain Reaction and Sequencing for Mycobacteria

Kennon Miller, MD; Susan M. Harrington, PhD; Gary W. Procop, MD

• *Context.*—New molecular diagnostic tests are attractive because of the potential they hold for improving diagnostics in microbiology. The value of these tests, which is often assumed, should be investigated to determine the best use of these potentially powerful tools.

***Objective.*—To investigate the usefulness of broad-range polymerase chain reaction (PCR), followed by sequencing, in mycobacterial infections.**

***Design.*—We reviewed the test performance of acid-fast bacilli (AFB) PCR and traditional diagnostic methods (histopathology, AFB smear, and culture). We assessed the diagnostic effect and cost of the unrestricted ordering of broad-range PCR for the detection and identification of mycobacteria in clinical specimens.**

***Results.*—The AFB PCR was less sensitive than culture and histopathology and was less specific than culture, AFB smear, and histopathology. During 18 months, $93 063 was spent on 183 patient specimens for broad-range PCR and DNA sequencing for mycobacteria to confirm one culture-proven *Mycobacterium tuberculosis* infection that was also known to be positive by AFB smear and histopathology. In this cohort, there was a false-negative AFB PCR for *M tuberculosis* and a false-positive AFB PCR for *Mycobacterium lentiflavum*.**

***Conclusion.*—Testing of AFB smear–negative specimens from patients without an inflammatory response supportive of a mycobacterial infection is costly and has not been proven to improve patient care. Traditional diagnostics (histopathology, AFB smear, and culture) should remain the primary methods for the detection of mycobacteria in clinical specimens.**

(*Arch Pathol Lab Med.* 2015;139:1020–1023; doi: 10.5858/arpa.2013-0705-OA)

Selection of the optimum tests for a specimen becomes more challenging as newer testing methods become increasingly available for the detection of microorganisms. Polymerase chain reaction (PCR)–based methods for microorganism detection and identification are attractive given the sensitivity and speed of many of these assays.[1,2] Broad-range PCR assays (ie, PCR assays that detect large groups of microorganisms) have been used successfully as an antecedent to DNA sequencing for the detection and identification of a variety of microorganisms.[3–5] This has been used after cultivation to identify microorganisms when traditional methods fail.[6] Additionally, it has been used directly on clinical specimens that contain evidence of infection (ie, microorganisms in an appropriate inflammatory response) when traditional methods of culture are unproductive.[5,7,8] However, the reported advantages of these assays such as speed and sensitivity make them prone to misuse. For example, broad-range PCR for bacteria, fungi, and mycobacteria has been used as a more expensive replacement for the inefficient practice of "pan-culture" that preceded it.[9] Unfortunately, this approach (which we will demonstrate) is equally unsound and significantly more expensive for the detection of mycobacteria in clinical specimens and should be reconsidered in an era of health care reform when cost-effective, evidence-based approaches are needed.

At our institution, broad-range PCR, followed by DNA sequencing, is a send-out test that may be requested for the detection of bacteria, fungi, and mycobacteria on multiple types of specimens, including tissue specimens. Requests for the detection of mycobacteria by broad-range PCR and DNA sequencing (hereafter referred to as acid-fast bacilli [AFB] PCR) are often made on specimens that are also submitted for histopathology and to the microbiology laboratory for AFB smear and mycobacteria culture. We sought to determine if the findings of histopathologic assessment (eg, granulomas) that can suggest mycobacterial infection, as well as the direct demonstration of AFB by special stains (ie, AFB smear and AFB staining of histologic sections), may be useful in predicting the usefulness of this

Accepted for publication December 2, 2014.
Published as an Early Online Release January 9, 2015.
From the Department of Molecular Pathology and Microbiology, Cleveland Clinic, Cleveland, Ohio.
The authors have no relevant financial interest in the products or companies described in this article.
The opinions expressed represent those of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention.
Reprints: Gary W. Procop, MD, Department of Molecular Pathology and Microbiology, Cleveland Clinic, 9500 Euclid Ave, Cleveland, OH 44195 (e-mail: procopg@ccf.org).

molecular test and possibly guiding its appropriate use. We were also interested in determining the cost and benefit (ie, the incremental value) of adding broad-range PCR and DNA sequencing for mycobacteria testing to specimens that were already submitted for histopathology, AFB smear, and mycobacteria culture.

MATERIALS AND METHODS

After institutional review board approval, a retrospective medical record review was conducted for all tissue specimens on which AFB PCR, histopathology, AFB smear, and mycobacteria culture had been performed between December 29, 2011, and June 26, 2013 (ie, 18 months). Specimens were excluded if results were not available for each of these 4 tests for comparison purposes. A test result was considered a true positive if it was positive by at least 2 methods (eg, AFB PCR positive and AFB smear positive). When a single positive test result was present that could not be corroborated by another method, then it was considered a false positive. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the 4 tests were determined by standard methods.

Histopathologic assessment of specimens was performed at the Cleveland Clinic by subspecialty anatomic pathologists. Hematoxylin-eosin staining was performed on all specimens. Staining for microorganisms was performed on specimens at the discretion of the attending pathologist. Final diagnoses were reviewed for the presence of granulomas and for the presence of AFB if Ziehl-Neelsen staining was performed. Histopathology specimens were considered definitively positive for the presence of mycobacteria if an appropriate inflammatory response was present and AFB were detected in tissues. Tissues with the presence of an appropriate inflammatory response but the absence of AFB in tissues were considered supportive of but not diagnostic for an infection by mycobacteria because a variety of conditions can result in granulomas. The inflammatory responses considered most supportive of a possible infection by mycobacteria were granulomas (necrotizing or nonnecrotizing) and pyogranulomatous inflammation. However, we recognize that mycobacteria are less commonly associated with a neutrophilic response or with the inflammatory pseudotumor in an immunocompromised host.

Tissue specimens submitted to microbiology were ground with sterile tissue grinders, digested, and decontaminated before acid-fast staining and culture. Fluorochrome (auramine O) staining was performed on all specimens sent for AFB culture. Positive fluorochrome stains were confirmed using a Ziehl-Neelsen stain. The specimens were inoculated into Mycobacteria Growth Indicator Tubes (Becton Dickinson Diagnostics, Sparks, Maryland) containing a modified Middlebrook 7H9 broth base and onto Middlebrook 7H11 agar (both from Becton Dickinson Diagnostics, Sparks, Maryland) with and without antibiotics. The presence of mycobacteria in positive cultures was confirmed with a Ziehl-Neelsen stain. Species identification was performed using pyrosequencing[10] or ribosomal RNA probes (Hologic-Gen-Probe, Inc, San Diego, California). The AFB cultures were held for 42 days to assess for growth. Results of the AFB smear and culture were recorded.

Fresh tissue, if available, was sent to a Clinical Laboratory Improvement Act–certified commercial vendor for AFB PCR. If fresh tissue was not available, then the formalin-fixed, paraffin-embedded block was sent. The methods used are proprietary. They performed DNA extraction and purification, followed by PCR for *Mycobacterium tuberculosis* complex and *Mycobacterium avium/Mycobacterium intracellulare* complex using primers targeting the heat shock protein 65 (*hsp65*) in a nested PCR assay. The other nontuberculous mycobacteria were screened by targeting multiple loci of the mycobacterial genome. Results of the PCR were recorded. If amplification occurred, then sequencing was performed, and species-level identification was provided. Finally, we examined the total cost of the AFB PCR for the number of specimens reviewed and, based on the positivity rate in the cohort, calculated the cost per clinically meaningful positive result.

RESULTS

Three hundred six specimens were sent for AFB PCR between December 29, 2011, and June 26, 2013. Histopathology, AFB smear, culture, and AFB PCR were performed on 183 of these specimens. The AFB PCR had been ordered at the time the tissue specimens were acquired as part of a larger protocol that included broad-range PCR and sequencing for bacteria and fungi. Much of this protocol was focused on endocarditis. The remaining 123 specimens lacked either histopathology (n = 94), AFB culture (n = 10), or both (n = 19). Of the 113 specimens lacking histopathology, 82 did not have histopathology ordered, 16 had only gross examinations, and 15 were cytology specimens.

The most common specimen sites were cardiovascular (n = 136) and bone, joint, and soft tissue (n = 27). The remaining specimen sites included brain tissue (n = 6), lung (n = 4), liver (n = 3), sinus (n = 2), lymph nodes (n = 2), genitourinary (n = 2), and spleen (n = 1). Of the 136 cardiovascular specimens, there were 121 valves, 10 vessel wall specimens (ie, aorta), 3 specimens associated with pacemakers, and 2 pericardial specimens.

Of the 183 tissue specimens that met the inclusion criteria, 9 patient specimens (4.9%) had at least 1 positive finding (Table). Granulomas were found in the biopsy specimens of 7 patients on histopathologic examination. All 7 cases had AFB stains performed, with 2 cases demonstrating AFB (ie, 2 patients were positive by histopathology). On clinical microbiology, the AFB smear was positive for 1 patient, whereas the AFB culture was positive for 3 patients. The AFB PCR was positive for 2 patients.

There were only 2 patients (patients 1 and 2 in the Table) who met the criteria for a true positive (ie, a positive test corroborated by a second positive test). Using the defined criteria, there was 1 false-positive PCR result (patient 3) and 1 false-positive culture result (patient 4). There were 5 patients in the study whose biopsy specimens contained granulomas but no evidence of disease caused by mycobacteria, as determined by AFB stain on tissue, AFB smear, and culture. These, as well as the false-positive culture and false-positive AFB PCRs, were investigated.

Patient 1 demonstrated granulomas on histopathologic examination and had AFB detected by histochemical stains. These findings were communicated to the clinical team on postoperative day 3. The microbiology specimen was positive for AFB on direct examination with fluorochrome staining completed on the day it was received by the laboratory. The patient was started on a 4-drug regimen for presumed *M tuberculosis* at that time because of a clinical suspicion of tuberculosis. The AFB PCR was positive for *M tuberculosis* on postoperative day 14. On postoperative day 20, *M tuberculosis* was identified from the culture.

A neck lymph node was excised from patient 2 that demonstrated granulomas on histopathology, with rare AFB detected on special staining. The specimen sent to microbiology was negative on direct examination (ie, a false-negative AFB smear), but *M tuberculosis* was identified by culture on day 15. The AFB PCR was negative (ie, a false-negative AFB PCR).

There was 1 false-positive AFB PCR test result. The AFB PCR from the mitral valve of patient 3 yielded *Mycobacterium lentiflavum*. Histopathologic assessment of the valve demonstrated bacterial endocarditis. Blood cultures, tissue cultures, and bacterial PCR revealed methicillin-resistant *Staphylococcus aureus*, and the patient was treated with 6

Patients With Positive Test Results						
		Histopathology			Microbiology Laboratory	
Patient No.	Site	Granulomas	AFB Stain	PCR Result	Smear	AFB Culture
1	Ethmoid sinus	Yes	Positive	*Mycobacterium tuberculosis* complex	Positive	*Mycobacterium tuberculosis* complex
2	Neck lymph node	Yes	Positive	Negative	Negative	*Mycobacterium tuberculosis* complex
3	Mitral valve	No	NA	*Mycobacterium lentiflavum*	Negative	Negative
4	Bone, L1–L2	No	NA	Negative	Negative	MAI complex
5	Liver mass	Yes	Negative	Negative	Negative	Negative
6	Subcarinal tissue	Yes	Negative	Negative	Negative	Negative
7	Elbow triceps tendon	Yes	Negative	Negative	Negative	Negative
8	Neck lymph node	Yes	Negative	Negative	Negative	Negative
9	Lung, left upper lobe	Yes	Negative	Negative	Negative	Negative

Abbreviations: AFB, acid-fast bacilli; L1–L2, lumbar spaces 1 to 2; MAI, *Mycobacterium avium/Mycobacterium intracellulare* complex; NA, not applicable; PCR, polymerase chain reaction.

weeks of vancomycin. The patient was not treated for *M lentiflavum* because it was considered a contaminant. The patient had improved at the last follow-up 3 months after surgery.

Similarly, there was 1 false-positive culture in patient 4 with vertebral osteomyelitis. Histopathology from a lumbar spine biopsy specimen demonstrated segments of fibrous tissue without inflammation. Bacterial and fungal cultures were negative, but an AFB culture demonstrated *M avium/M intracellulare* complex. Bacterial, fungal, and AFB PCR testing were negative. The impression of the clinical team was that the *M avium/M intracellulare* complex was a contaminant. This patient subsequently developed aortic valve endocarditis requiring replacement. Cultures and PCR from the valve were negative. A single blood culture demonstrated viridans streptococci, but its significance was uncertain. Cultures from a subsequent debridement and fusion of the lumbar spine were negative. The patient was treated empirically with vancomycin and ceftriaxone, followed by oral suppressive clindamycin, with clinical improvement at the last follow-up 1 year after cardiac surgery.

There were 5 patients (patients 5, 6, 7, 8, and 9) in whom the only suggestive finding was histopathology demonstrating granulomas. Patient 5 and patient 6 were treated for fungal infections. Bacterial etiologies were clinically suspected in patient 7 and patient 8. The granulomas in patient 9 were found in association with myeloid sarcoma. Special histochemical stains for organisms were negative, and all cultures and PCR studies were negative.

The sensitivity, specificity, PPV, and NPV, respectively, for the tests studied were as follows: histopathology (100%, 100%, 100%, and 100%), AFB smear (50%, 100%, 100%, and 99%), mycobacteria culture (100%, 99%, 67%, and 100%), and AFB PCR (50%, 99%, 50%, and 99%). It is recognized that the sensitivity and PPV are likely affected by the very low number of positive specimens in this study. If a supportive inflammatory response (ie, granulomas in tissue) was used as an inclusion criterion, then only 7 specimens would have been sent for PCR, resulting in a savings of $152 053. If such criteria were used, the sensitivity, specificity, PPV, and NPV, respectively, for the tests studied would be as follows: histochemical stain for AFB (100%, 100%, 100%, and 100%), AFB smear in microbiology (50%, 100%, 100%, and 83%), mycobacteria culture (100%, 100%, 100%, and 100%), and, AFB PCR (50%, 100%, 100%, and 83%). This assessment of only those specimens with an appropriate inflammatory response is limited by the small number of specimens meeting this criterion. However, it clearly demonstrates that such criteria could be used to triage which specimens are more likely to yield meaningful results (ie, increase the pretest probability).

The cost of the AFB PCR was $509 per specimen. During the 18-month period reviewed, $155 613 was spent on the 308 specimens sent for AFB PCR. Of the 183 specimens reviewed, the cost was $93 063. The cost per true positive in the cohort reviewed (ie, 183 specimens) was $93 063 per true positive. If only the 7 specimens that contained granulomas were sent for testing, then the cost per true positive would be reduced to $3560 per true positive.

DISCUSSION

The advent of molecular diagnostics holds promise for assays that, in many instances, are better and faster than traditional methods. However, it has been our experience that molecular tests are often deemed superior to traditional microbiologic assays on the basis that they are molecular and without consideration of the performance characteristics of the various tests. There has been unrestricted access to broad-range PCR and DNA sequencing for bacteria, mycobacteria, and fungi at our institution. In this study, we limited our review to those cases that included histopathology, AFB smear, and culture results in the expectation that results from the AFB smear and histopathology may provide guidance for future studies.

Each of the studies reviewed (ie, histopathology, AFB smear, culture, and AFB PCR) offers certain advantages. For tissue specimens, histopathology is particularly useful because it can define 1 or more underlying pathologic processes, in this case the inflammatory response and the presence or absence of AFB. The assessment for granulomas and subsequent AFB stains can be performed and interpreted relatively quickly (ie, within days). The AFB smear performed in microbiology provides the most rapid information to the clinician regarding the presence of AFB. Although culture is not rapid, it is sensitive and provides a viable organism for identification and susceptibility studies. We sought to investigate the contribution of broad-range PCR for mycobacteria and DNA sequencing applied directly to the clinical specimen and to compare it with the available diagnostic tools, particularly given the expense of the newer assay.

The main advantages purported for PCR-based assays are improved sensitivity and decreased time to organism detection. Although there were only 2 confirmed infections caused by mycobacteria in this study, the AFB PCR did not offer improved sensitivity over traditional methods. Disappointingly, of the 2 culture-confirmed patients, only patient 1 was AFB PCR positive, and for this patient the AFB smear and histopathology results were available long before the molecular result was received. Others have reported that the nucleic acid–based amplification assays have decreased sensitivity for AFB smear–negative specimens.[11,12] The AFB PCR false negative was in patient 2, who was also AFB smear–negative and had only a few AFB detectable on histopathology examination, which likely explains this result.

In 175 of the patient specimens studied (95.6%), there were neither granulomas nor evidence of AFB on smears or cultures; the AFB PCR did not offer any useful clinical information for these patients. Otherwise stated, there were no undiagnosed instances of disease caused by mycobacteria that were discovered by the AFB PCR. Instead, the only specimen with negative histopathology, culture, and AFB smear, but positive AFB PCR, was demonstrated to be a false positive (ie, a standard bacterial cause of endocarditis was proven by culture, Gram stain, and histopathology) and was considered as such by the clinical team. Given the low prevalence of mycobacterial infections in this study, it is not surprising that a positive AFB PCR result may be classified as a false positive. The low prevalence in this study is a reflection of our patient population. It is also likely related to the fact that the majority of specimens were from cardiovascular sites, which are rarely involved by mycobacterial infections. It would be expected that the PPV of the test would be improved in an area with a higher prevalence of tuberculosis. However, we would still contend that an assessment of the histopathologic and AFB smear results should be used as a means to triage specimens for testing because this would increase the pretest probability.

These results suggest that AFB PCR does not offer any substantial benefit if ordered as part of a protocol at the time of specimen collection. Additionally, histopathology, AFB smear, and culture are more cost-effective in screening for mycobacteria. Accordingly, we suggest using histopathologic assessment and AFB smear results to guide the use of AFB PCR. If a specimen has a positive AFB smear or if histopathology demonstrates granulomas with AFB, then broad-range PCR for mycobacteria, followed by DNA sequencing, may add value in determining the identity of the causative species before culture results are available. However, even with the limited number of positive specimens in this study, 1 of the 2 patients whose specimen demonstrated granulomas with AFB on histopathology and a positive AFB culture (patient 2) was negative by AFB PCR. In high-prevalence settings, AFB PCR may be useful if the clinical suspicion (ie, pretest probability) is high; however, it would be expected that histopathology, AFB smear, and culture would also be informative in these patients.

This approach, using histopathologic assessment and AFB smear results to guide AFB PCR use, would not delay species-level identification by AFB PCR significantly. A positive AFB smear should be reported as soon as the specimen is processed by the microbiology laboratory, and AFB PCR could be requested at that time. If the AFB smear is negative, histopathologic assessment for granulomas and AFB staining could be reported within 1 to 2 days, and, if present, the AFB PCR could be added at that time. The clinical scenario for the one true-positive AFB PCR case herein (patient 1) essentially followed this algorithm because histopathologic assessment and AFB smear were completed quickly. The clinician's acuity led to appropriate and timely treatment of the patient, approximately 10 days before the AFB PCR identified the infectious species. Obviously, the delay in AFB PCR is dependent on whether this test is performed in-house or as a send-out test.

Each assay, molecular or traditional, has strengths and limitations that should be acknowledged when the tests are being ordered. As long recognized, the posttest likelihood of a particular result rests largely on the pretest probability.[13] Our results suggest that AFB PCR would be most judiciously used as a supplemental test when there is a high pretest probability of mycobacterial infection (ie, histopathology with an appropriate inflammatory response with AFB present, a positive AFB smear, or clinical suspicion). The AFB PCR was less sensitive than culture and histopathology and was less specific than culture, AFB smear, and histopathology in this study. The cost of $93 063 to detect the one true-positive case, which was already detected by traditional methods, is a price that will not likely be supported in an era of health care reform. In this study, the use of an expensive molecular diagnostic test resulted in substantial unnecessary costs in our health care system. Our findings support the continued use of traditional methods (ie, histopathology, AFB smear, and culture) as the primary means for assessing clinical specimens for the presence of mycobacteria. The use of broad-range PCR and DNA sequencing should be reserved for those rare instances in which mycobacteria are demonstrated to be present and species-level identification would otherwise not be possible.

This work was supported by cooperative agreement U47CI000831 with the Cleveland Clinic, with funds provided in whole or in part from federal funds from the Division of Laboratory Science and Standards, Office of Surveillance, Epidemiology, and Laboratory Services, Centers for Disease Control and Prevention.

References

1. Louie L, Goodfellow J, Mathieu P, Glatt A, Louie M, Simor AE. Rapid detection of methicillin-resistant staphylococci from blood culture bottles by using a multiplex PCR assay. *J Clin Microbiol*. 2002;40(8):2786–2790.

2. Zucol F, Ammann RA, Berger C, et al. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. *J Clin Microbiol*. 2006;44(8):2750–2759.

3. Petti CA. Detection and identification of microorganisms by gene amplification and sequencing. *Clin Infect Dis*. 2007;44(8):1108–1114.

4. Sontakke S, Cadenas MB, Maggi RG, Diniz PP, Breitschwerdt EB. Use of broad range 16S rDNA PCR in clinical microbiology. *J Microbiol Methods*. 2009;76(3):217–225.

5. Böttger EC, Teske A, Kirschner P, et al. Disseminated "*Mycobacterium genavense*" infection in patients with AIDS. *Lancet*. 1992;340(8811):76–80.

6. Pottumarthy S, Schapiro JM, Prentice JL, et al. Clinical isolates of *Staphylococcus intermedius* masquerading as methicillin-resistant *Staphylococcus aureus*. *J Clin Microbiol*. 2004;42(12):5881–5884.

7. Rampini SK, Bloemberg GV, Keller PM, et al. Broad-range 16S rRNA gene polymerase chain reaction for diagnosis of culture-negative bacterial infections. *Clin Infect Dis*. 2011;53(12):1245–1251.

8. Relman DA, Schmidt TM, MacDermott RP, Falkow S. Identification of the uncultured bacillus of Whipple's disease. *N Engl J Med*. 1992;327(5):293–301.

9. Wolk D, Mitchell S, Patel R. Principles of molecular microbiology testing methods. *Infect Dis Clin North Am*. 2001;15(4):1157–1204.

10. Tuohy MJ, Hall GS, Sholtis M, Procop GW. Pyrosequencing as a tool for the identification of common isolates of *Mycobacterium* sp. *Diagn Microbiol Infect Dis*. 2005;51(4):245–250.

11. Nahid P, Pai M, Hopewell PC. Advances in the diagnosis and treatment of tuberculosis. *Proc Am Thorac Soc*. 2006;3(1):103–110.

12. Bergmann JS, Woods GL. Clinical evaluation of the Roche AMPLICOR PCR Mycobacterium tuberculosis test for detection of M. tuberculosis in respiratory specimens. *J Clin Microbiol*. 1996;34(5):1083–1085.

13. Deeks JJ, Altman DG. Diagnostic tests, 4: likelihood ratios. *BMJ*. 2004;329(7458):168–169.