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Next-Generation Sequencing Supports Targeted Antibiotic Treatment for Culture Negative Orthopedic Infections

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The isolation of an infective pathogen can be challenging in some patients with active, clinically apparent infectious diseases. Despite efforts in the microbiology lab to improve the sensitivity of culture in orthopedic implant-associated infections, the clinically relevant information often falls short of expectations. The management of peri-prosthetic joint infections (PJI) provides an excellent example of the use and benefits of newer diagnostic technologies to supplement the often-inadequate yield of traditional culture methods as a substantial percentage of orthopedic infections are culture-negative. Next-generation sequencing (NGS) has the potential to improve upon this yield. Bringing molecular diagnostics into practice can provide critical information about the nature of the infective organisms and allow targeted therapy in these otherwise challenging situations. This review article describes the current state of knowledge related to the use and potential of NGS to diagnose infections, particularly in the setting of PJIs.

Keywords. antibiotic stewardship; orthopedic infections; next-generation sequencing; metagenomics; clinical microbiology.

Culture has been regarded as the *gold standard* for diagnosing many infectious diseases. However, there are several issues with traditional culture platforms [1, 2]. One important limitation of culture is that many organisms, particularly fastidious and anaerobic organisms, remain difficult to grow with standard microbiology laboratory techniques. Estimates of the proportion of typically unculturable bacteria range from 80% to >99% of the predicted total bacterial organisms in existence. Still, it remains unclear what proportion of clinically relevant microorganisms are fully undetectable by modern culture-based approaches [3]. Thus, relying on the metabolic activity of pathogens for isolation, culture fails to identify organisms that are in a viable but not culturable state (VBNC). Traditional cultures often have low yield when organisms associated with implant-related infections exist in a biofilm on an implant surface [4–6]. As a result, up to 42% of orthopedic device-related infections are culture-negative [7, 8], likely due to organisms requiring a longer incubation time, in vitro suppression from antibiotic treatment, and lack of enriched culture medium [9, 10].

Peri-prosthetic joint infections (PJI) are a serious complication of total joint arthroplasty and are associated with high morbidity [11, 12]. An accurate and timely diagnosis of PJI, including identification of the infecting pathogen, is necessary for optimal antibiotic treatment. Despite extensive efforts, cultures typically have a high false-negative rate, making it difficult for the surgeon and infectious disease clinician to manage these cases. Studies have shown that culture-negative PJI is associated with poor outcomes [13–15]. Identification of the causative organism is one of the primary predictors for success in diagnosing and treating PJI [16, 17]. A retrospective review of over 200 patients who had surgery to treat culture-negative PJI found that ~30% of patients failed treatment >1 year of follow-up, with many patients requiring salvage procedures [13]. In a multivariate analysis, Mortazavi et al showed that culture-negative PJI was a predictor of failure for 2-stage exchange arthroplasty of the knee (odds ratio [OR]: 4.5; 95% confidence interval [CI]" 1.3–15.7) [14]. The orthopedic and medical communities need technologies that improve upon traditional culture in identifying the infecting organism.

The use of molecular techniques to detect infective pathogens in challenging infections, such as PJI, is not new. Numerous technologies including enzyme-linked immunosorbent assay (ELISA)-based hybridization, fluorescence-based real-time detection, liquid or solid phase microarray detection, and matrix-assisted laser desorption/ionization-time of

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flight mass spectrometry (MALDI-TOF MS) are molecular diagnostic methods that have improved upon conventional culture methods, decreasing time to pathogen identification, optimizing antibiotic therapy, and improving patient outcomes. These molecular methods, however, can detect only a limited number of specific pathogens, may have limited taxonomic resolution, or may have limitations on scaling to large sample volumes. Separate from these approaches effort has been concentrated chiefly around methodologies leveraging polymerase chain reaction (PCR) [18, 19]. PCR relies on amplifying pathogen DNA targeting conserved genes. PCR can detect a predetermined and finite group of organisms based upon the primers chosen for the assay or can be tailored to detect a broad range of organisms. The primary advantages of PCR over traditional culture are its high sensitivity regardless of cell viability, prior antibiotic use, or fastidious growth.

In contrast to these platforms, next-generation sequencing (NGS) is a more comprehensive technology that usually leverages PCR in the sample preparation stage. As the name suggests, NGS relies on sequencing the available genetic material in a given sample. The sequences are then queried against a curated database of gene sequences from different species, and the best highly similar match is used to assign species to the query sequence. With the rapid decline in the cost of sequencing over the last few years, NGS is steadily finding a place in clinical practice and already beginning to play a critical role in detecting infective organisms [20]. Table 1 lists some benefits and weaknesses of NGS compared to culture for orthopedic infections. Here we will review molecular methods that are used or are being evaluated for use in a clinical setting, including (quantitative real-time) PCR and NGS methods. We will also describe the role of NGS in PJI.

Table 1. Benefits and Weaknesses of NGS and Culture for Orthopedic Infections

	Current Microbiology Techniques (Culture)	Next-Generation Sequencing (NGS)
Benefits	• Widely available and can be performed in any microbiology lab with basic equipment	• Higher sensitivity and specificity than culture • Decreased time for some pathogen identification (especially in slow growing organisms such as *C. acnes*, AFB, and fungi) • Identification of organisms present, independent of prior antibiotic use
Weaknesses	• Fails to identify organisms that are viable but not culturable (VBNC) • Requires special media and condition for specific microorganisms • There is an increased risk of contamination especially if prolonged incubation is required • Large fraction of microbes cannot be grown with culture	• May be confounded by presence of host DNA in the sample • Requires highly specialized equipment, trained technicians, and bioinformatics expertise is needed

Abbreviations: AFB, acid-fast bacteria; NGS, next-generation sequencing.

AN OVERVIEW OF SEQUENCE-BASED DIAGNOSTIC APPROACHES

PCR-based applications such as real-time or quantitative PCR (qPCR) were recognized more than 20 years ago to offer enhanced sensitivity and scalability compared to culture-based methods [21, 22], including in the context of orthopedic infection [18]. Referred to as PCR in medical settings, qPCR relies on pre-validated assays to amplify pathogenic DNA by targeting a gene sequence that is ideally conserved within the target group and uniquely discriminating from off-target organisms/genes [20]. To screen for multiple targets, PCR panels and/or multiplexed assays must be validated with generally one assay per unique target. PCR testing is now routinely used in clinical laboratory diagnostics for rapid and targeted amplification of specific organisms, antimicrobial resistance (AMR) associated genes, and microbial toxins and can be paired with a cDNA reverse transcriptase reaction for RNA targets (eg, severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2] testing).

Since its introduction, NGS has been used extensively to catalog the human microbiome in health and disease, with particular attention to differing microbial taxonomy, phylogeny, and function. The most common applications of NGS are amplicon sequencing and shotgun metagenomics. The latter is sometimes referred to more briefly as mNGS [23]. NGS has been used for outbreak tracking and surveillance programs, mutation detection, oncology, identification of multiple pathogens in a single sample, and AMR testing, among many other applications [20].

It is difficult to estimate the current usage of NGS in clinical infectious diseases because these endeavors are dispersed among third-party private labs and a few large institutions. Still, the likely most common microbial profiling approaches are variants of amplicon sequencing. Amplicon sequencing may also be referred to as metabarcoding (simply) NGS or may more accurately reference the specific marker used (eg, 16S/ITS), among other names. Amplicon sequencing uses PCR amplification products (amplicons) of functionally conserved marker gene fragments to identify bacteria, archaea, fungi, and mycobacteria from a single sample, depending on the marker gene chosen. Through increased use of amplicon sequencing with clinical specimens, multiple studies have reported increased incidence of difficult-to-culture organisms such as anaerobes, increased ability to detect less common microbes, and an increased number of polymicrobial specimens [24].

One of the most studied target genes for amplicon sequencing is the bacterial 16S rRNA gene, which is universally conserved among bacteria and is capable of enhanced sensitivity

in detecting and identifying bacteria [24]. Before sequencing, PCR is conducted using oligonucleotide primers complementary to conserved regions of the 16S gene universal to bacteria and extend through variable regions that contain informative variation used to make taxonomic identification. The identification process is highly dependent on the primer region, primer design, bioinformatic processing, and quality of the taxonomic reference database, necessitating rigorous validation to ensure all these components and more are operating as intended. The internal transcribed spacer (ITS) region serves as a similar universal fungal target.

Shotgun metagenomic methods (or mNGS) can use long-read or short-read sequencing technologies to sample all genomic material within a specimen [20, 25]. mNGS uses fragmented DNA directly from the sample, sequenced and aligned back to reference genome databases using more advanced bioinformatic and computationally intensive methods. By capturing the entire genetic complement of the sample, species or even strain-level calls can be more confidently made, and further information can be extracted. A common goal of this method is to characterize further the AMR profile of a microbiological specimen, including all known AMR-associated genes and possibly mutations conferring resistance, as well as other accessory functions [20].

TECHNICAL HURDLES FOR NGS IN THE LABORATORY

Although mNGS can provide the most information using the genomic content of the sample, the method poses unique challenges from PCR and even amplicon sequencing, which have generally prohibited use outside of research. It is significantly confounded by the presence of host DNA in the sample, thus requiring a significant increase in sequencing depth to overcome both host DNA and adequately characterize the microbes present. For example, Kalan et al [26] investigated the metagenomic profiles of chronically infected wound specimens. They sequenced them to a median depth of 144M reads/sample, although an average of 97.5% of those reads were human and discarded. After filtering, the authors were left with a median of 2.4M microbial reads per sample and further estimated that 10M microbial reads would be needed to characterize the microbial communities of those particular samples sufficiently. For comparison, between 1000 and 10 000 reads in the same specimen type using targeted 16S rRNA sequencing has been historically adequate for describing unique bacteria in infected tissue [27]. To overcome these barriers, recent work has shown promise in using host DNA depletion, or cell-free DNA approaches in a select few specimen types, to achieve more favorable host: microbe sequencing ratios. Due to the enrichment of target DNA inherent to amplicon sequencing, this method will likely remain more broadly applicable to various sample types with less specimen-specific optimization, as may be needed for mNGS to be practically applied in infectious diseases [28].

NEXT-GENERATION SEQUENCING (NGS): AVAILABLE EVIDENCE

In 2016, NGS was used at Thomas Jefferson University Hospital on a patient who had undergone total knee replacement 4 years earlier. The patient had an underlying immunodeficiency and developed severe pain and swelling at the knee joint. The condition of the knee continued to worsen, and the patient developed systemic sepsis and required admission to the intensive care unit. All samples sent for culture, including blood and synovial fluid, with prolonged incubation, had been negative. On the other hand, NGS detected the gene-specific for *Streptococcus canis* in both the synovial fluid and tissue cultures [29]. Interestingly, the source of infection in the patient was a pet dog that had been licking the superficial scratch around the knee that became infected. With the help of NGS, the authors could link the oral transmission of a pathogen from a pet to an orthopedic device.

Numerous published studies have demonstrated the promising role of NGS in detecting infective pathogens in orthopedic infections. In culture-negative cases, NGS can detect infective organisms from the synovial fluid or tissue samples and identify unusual microorganisms. Wang et al found that in samples from 12 culture-negative PJI patients, mNGS detected many rare pathogens and fastidious bacteria, such as *Mycoplasma hominis* (3 cases), *Finegoldia magna*, and *Parvimonas micra* [30]. Furthermore, in 49 PJI patients, Huang et al showed that the sensitivity of mNGS for diagnosing PJI was significantly higher than culture (95.9% vs 79.6%), and mNGS was useful in identifying pathogens that typically require special culture conditions, such as *Mycoplasma* and *Mycobacterium* [31]. Thoendel et al compared sonicate fluid culture results to mNGS analysis from 408 resected hip and knee arthroplasties. Compared to sonicate fluid culture, NGS identified known pathogens in 94.8% (109/115) of culture-positive PJIs, with more potential pathogens detected in 9.6% (11/115). New potential pathogens were detected in 43.9% (43/98) of culture-negative PJIs, 21 of which had no other positive culture sources from which these organisms had been detected [32].

Despite studies showing the high sensitivity of NGS, it has not been used much in orthopedic culture-negative clinical practice. Studies are warranted to show the specificity of NGS to correlate with better patient outcomes compared to conventional diagnostic methods. Furthermore, with such high sensitivity, there needs to be a better understanding of the clinical significance of identifying unusual organisms that may not necessarily be involved in the disease process. Additionally, NGS requires highly specialized equipment, trained technicians, and bioinformatics expertise to ensure effective sequencing runs, which may not be practical in many clinical settings.

NEXT-GENERATION SEQUENCING AND ANTIBIOTIC STEWARDSHIP

A concern regarding NGS-guided antibiotic selection is that NGS reports may identify multiple organisms in the case of an infection, further supporting the administration of broad-spectrum or otherwise unnecessary antibiotics. Antibiotic therapy, regardless of if using NGS or culture, should not be based on the diagnostic result alone, but in the context of suspected infection and in accordance with accompanying criteria where established. Tarabichi et al reported on 11 cases of culture-negative PJI in which NGS identified an organism in 9 cases (81.8%) [33], suggesting better use of targeted antibiotics. In another observational study where 79.3% of patients failed after a 2-stage exchange arthroplasty, the infective organism(s) were the same as those identified by NGS at the time of first operation (resection arthroplasty) that was not treated [34]. Furthermore, Wang et al used mNGS to evaluate the efficacy of targeted antibiotics for the treatment of culture-negative PJI compared to empirical antibiotic therapy, resulting in a better infection control rate, lower antibiotic-related complications, and a shorter duration of antibiotic treatment when targeted antibiotics were used according to mNGS results [30]. Recent work involving over 140 000 urinary tract infections and 7000 wound infection specimens similarly found that mNGS could often detect the infecting organism detected at follow-up earlier in cases of reinfection. The authors estimated that when antibiotic selection accurately covered the infection profile described by mNGS, those patients had a 70–74% lower recurrence risk than those with poor mNGS-determined antibiotic coverage [35]. Though further studies are warranted to evaluate the clinical outcomes and impact on antibiotic practices, NGS methodologies offer advantages in increased identification of infective organisms. They may enhance targeted antibiotics' administration, decreasing antimicrobial resistance rates.

NEXT-GENERATION SEQUENCING: CHALLENGES REMAINING

Current implementations of NGS have been met with skepticism. They have raised valid points of concern, ranging from practical challenges in implementation to technical problems and the need for further studies and optimization. Concerning practical challenges, the turnaround time for reporting and interpretation of complex reports are two examples where specific service providers will vary and are focus areas of improvement. A single Illumina Miseq runs can take between 17 and 56 hours, depending on the flow cell and parameters used, without factoring in shipping or processing time [20]. One interesting solution, albeit PCR, to increase testing speed involved adding an automated *Clostridioides difficile* PCR testing procedure to a hospital EMR. The automated testing protocol was associated with a 49% decreased nosocomial infection rate and a 26% reduction in mean hospital stay [36]. Likewise, the effectiveness of NGS in practical applications may be improved by innovative strategies that lead to earlier testing, and NGS providers will likely continue to focus on increased workflow efficiency.

Furthermore, currently, there are no FDA-cleared or approved tests using NGS technologies for infectious diseases. The current guidelines for NGS diagnostics have been established mainly in the oncology field, which differs from infectious diseases. Large clinical studies showing the clinical outcomes with NGS compared to culture are warranted.

Early use of NGS may help to avert unnecessary second or third-line diagnostic investigations and establish a diagnosis. In doing so, the cost savings associated with an early diagnosis may offset the NGS testing costs. In practice, the case for using in-house NGS as part of an antimicrobial stewardship program (ASP) may be complex due to the high initial cost of the equipment and uncertainty about the significant long-term savings that may result from the use of NGS. Studies demonstrating the cost-effectiveness of NGS as part of a stewardship approach with the ASP team, microbiology lab, and treating clinicians may be warranted.

More technically, NGS reporting depends on specimen-specific issues and laboratory bioinformatics. Davis et al discussed the issue of specimen contamination contributing to false-positive calls [37]. This issue may be exacerbated in low biomass samples and requires rigorous contamination monitoring protocols [37]. Orthopedic-related specimens collected from joints and implant devices are among those often considered low biomass and, as a result, may have a lower specimen positivity rate compared to other infectious sites [24].

NGS may still miss the identity of infective organisms in up to 30% of culture-negative cases [34]. NGS may indeed isolate organisms in patients and sites without clinical infection. This is explained for the most part by our current understanding of the human microbiome. Recent studies have shown that many sites in the body, including joints such as the shoulder, hip, and knee, have a distinct microbiome [38]. These organisms live in a state of equilibrium without causing damage to the host. A disruption of this equilibrium may lead to dysbiosis with resultant local tissue damage, eliciting an inflammatory response. The presence of non-pathogenic organisms detected by NGS compels us to seek a strategy to differentiate between symbiotic or non-pathogenic colonization of microorganisms and active pathogens. Clinical correlation is the standard here, but at least one diagnostic lab offering NGS technology for orthopedic samples has included inflammatory markers in their testing to assist in detecting a potential pathogenic process (MicroGenDX, OrthoKEY®).

NEXT-GENERATION SEQUENCING: HERE TO STAY

A report by the American Society for Microbiology and the American Academy of Microbiology stated that "Next-generation sequencing (NGS) has the potential to dramatically

revolutionize the clinical microbiology laboratory by replacing current time-consuming and labor-intensive techniques with a single, all-inclusive diagnostic test" [39]. NGS has also been recognized by the World Health Organization [40]. The available data from numerous fields in medicine and our experience in orthopedics over the last 5 years support the notion stated so clearly by the microbiology community. Notably, this technology's effective and optimal use requires partnerships between clinicians, infectious disease practitioners, clinical microbiologists, and pharmacists. Training these individuals, including bench-level technologists, in the principles and concepts of molecular technology, especially NGS, provides a value-added opportunity to impact patient care by generating clinically actionable results. NGS is here to stay and plays a critical role in identifying infecting organisms that have escaped the traditional and often inadequate culture techniques.

CONCLUSION

Our paper adds to the growing body of information regarding the diagnostic science behind NGS and its clinical application in the setting of PJI. The use of NGS to enhance the sensitivity of culture in disease models where it is typical to have a low culture yield represents an essential addition to clinical infectious disease management. In addition to PJI, implementing this technology for other typically low-yield culture models such as culture-negative endocarditis, neutropenic fever, and chronic urinary tract infections may prove beneficial. Outcome studies are warranted if there is to be widespread adoption of NGS.

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