Current Concepts Review

Application of Nucleic Acid-Based Strategies to Detect Infectious Pathogens in Orthopaedic Implant-Related Infection

Emily Ann McClure, PhD, Paul Werth, PhD, Benjamin Ross, PhD, and Ida Leah Gitajn, MD, MS

Investigation performed at Dartmouth College, Hanover, New Hampshire, and Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire

- Implant-associated infection in orthopaedic surgery remains an enormous and largely unsolved clinical problem with a high rate of persistent or recurrent infection. This may be due, at least in part, to the potential for underdiagnosis by traditional microbial culture or the potential for culture to incompletely identify the microbial species present.
- Nucleic acid-based diagnostic techniques, focused on using the diagnostic information contained in DNA or RNA to identify microbial species, have been developing rapidly and have garnered escalating interest for both clinical and research applications.
- Commonly applied techniques include end-point polymerase chain reaction (PCR), quantitative PCR, Sanger sequencing, and next-generation sequencing. Understanding the specific strengths and weaknesses of each technique is critical to understanding their utility, applying the correct assessment strategy, and critically understanding and interpreting research.
- The best practices for interpreting nucleic acid-based diagnostic techniques include considering positive and negative controls, reads per sample, detection thresholds (for differentiating contaminants from positive results), and the primer set or targeted regions.

Implant-associated infection in orthopaedics remains a largely unsolved clinical problem with unacceptably high rates of treatment failure requiring reoperation, with rates exceeding 30%[1-3]. The consequences are devastating, with risk of recurrence, chronic dysfunction, amputation, and death in both trauma and arthroplasty populations[4-11]. Current treatment strategies focus on systemic antibiotics targeted against pathogens identified via culturing in association with surgical debridement and removal of implants. However, this treatment strategy has an unacceptably high rate of failure, which is likely due, at least in part, to issues with traditional culturing methods that may miss clinically relevant microbial species. Culture-negative infection and/or infection with incomplete identification of infecting species may result in inadequate antibiotic coverage, which very likely contributes to recurrence. This is clearly reflected in the inferior outcomes and higher recurrence rates associated with culture-negative infection compared with infections with identified microbial species[12].

Microbiological culture-based strategies have serious limitations, despite their status as the gold standard. Culture yields negative results in 7% to 50% of periprosthetic joint infection cases[13-16] and 30% of fracture-related infection cases[2,17], and there is concern that culture yields, even when positive, may be incomplete. This is related to several issues. First, traditional culturing methods are biased toward organisms that thrive under nutritional, atmospheric, and physiological conditions employed by diagnostic laboratories (common culture challenges reviewed by Lewis et al.[18]), which are different from physiological conditions that exist in implant-associated infection. Several studies have demonstrated that culture results insufficiently represent

Disclosure: The **Disclosure of Potential Conflicts of Interest** forms are provided with the online version of the article (http://links.lww.com/JBJS/H420).

the entirety of bacterial communities in infected wounds[19-21]. Second, traditional culture is biased toward planktonic free-floating microbes, compared with biofilm-based microbial communities. This likely results in a culture yield that misses the most important species for infection recurrence[19-22]. Third, some microbes flourish only when a second species is also present (polymicrobial cultures)[23-28]. For obligately polymicrobial infections, traditional culturing methods may fail to isolate causative pathogens. Fourth, culture is associated with a 3 to 10-day delay until the identification of the species. Lastly, there is no quantitative information with regard to the relative bioburden and spatial arrangement of microbial species alone and in combination.

Based on these issues, culture-independent molecular diagnostic techniques have been developing rapidly and have garnered escalating interest for both clinical and research applications[29,30]. A subset of molecular diagnostic techniques focus on diagnostic information contained in nucleic acids (NAs) (Table I). The benefits arising from the sensitivity of NA-based strategies may be tempered by the errors resulting from improperly handling specimens or interpreting data. As NA-based diagnostic techniques become more mainstream, it is critical for orthopaedic surgeons to become facile with the nuances associated with these diagnostic tools. Therefore, in this review, we aim to provide a comprehensive overview of NA-based analysis strategies and review important caveats and best practices around applying or interpreting NA sequencing-based techniques.

NA-Based Analysis Techniques

NA-based microbial assessment strategies, based on deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), may fill the gap between characterizing the most abundant microorganisms and the most clinically relevant microorganisms. Understanding the steps in gene expression is critical to understanding sequencing-based technology. Cells replicate DNA by separating successive small regions of the DNA into 2 single strands. A polymerase reads the single-stranded DNA and adds paired bases to prepare 2 identical double-stranded DNA molecules. Active cells transcribe DNA into RNA in a similar manner, but, instead of copying the entire sequence, they transcribe only a targeted region, resulting in a short strand of single-stranded RNA. Ribosomes bind the resulting messenger RNA (mRNA) and translate its sequence into amino acids to create a protein. Because the function of ribosomes is so essential, their binding abilities are highly conserved across all living organisms. Ribosomal RNA (rRNA) consists of highly conserved binding sites interspersed with hypervariable regions, which can

TABLE I Comparison of Molecular Strategies and Their Applications*

General Technology	Chemistry	Quantitative	Multiplex	Output	Other Names
End-point PCR	PCR			Amplicon	UMD-Universal PCR, rapid ribosequencing
	PMA PCR			Amplicon	
	ddPCR (Bio-Rad Laboratories)	X	X	Amplicon	
	PCR-DGGE		X	Amplicon	
	RFLP		X	Amplicon	
	ESI-MS		X	Amplicon	
qPCR and RT-PCR	DNA-binding dyes	X		Amplicon	
	TaqMan (Thermo Fisher Scientific)	X	X	Amplicon	
	FRET	X	X	Amplicon	
	Molecular beacon	X	X	Amplicon	
	Hybridization probe	X	X	Amplicon	
	MGB Eclipse probe (IDT)	X	X	Amplicon	
	Amplifluor (Sigma-Aldrich)	X	X	Amplicon	
	Scorpion primer (Millipore Sigma)	X	X	Amplicon	
	LUX primer (Invitrogen)	X	X	Amplicon	
	BD QZyme (BD Biosciences)	X	X	Amplicon	
Sanger sequencing	Chain termination			Single sequence	
NGS	Various	X	X	Multiple sequences	Deep sequencing, high-throughput sequencing

*PMA = propidium monoazide, dd = droplet digital, DGGE = denaturing gradient gel electrophoresis, RFLP = restriction fragment length polymorphism, ESI-MS = electrospray ionization mass spectrometry, and FRET = fluorescence resonance energy transfer.

be used to identify organisms at various taxonomic levels[31]. The 16S rRNA gene is a prokaryote-specific sequence that encodes the rRNA component of the ribosome. Sequencing the hypervariable regions of the 16S rRNA gene in DNA allows the identification of bacterial DNA[32]. There are several techniques that take advantage of these processes to identify pathogens, and each has unique advantages and disadvantages (Table II).

NA Extraction

Extraction methods are designed to separate NAs from other materials in a sample (cell debris, proteins, lipids). Extraction protocols begin with cell lysis to release NAs into solution. Subsequent steps include protein precipitation, lipid separation, and salt removal to produce a sample containing concentrated NAs with minimal impurities[33].

End-Point Polymerase Chain Reaction (PCR)

PCR is a technique of amplifying DNA outside the cell[34]. The basic PCR technique requires template DNA, primers, free nucleotides, and DNA polymerase. The reaction mix is heated to melt double-stranded DNA into 2 single strands. The mix is then cooled to allow annealing of primers to targeted sites. Primer sets are designed to include a forward and a reverse

TABLE II NA-Based Analysis Techniques: Advantages and Disadvantages

Technique	Basic Principle	Advantages	Disadvantages
End-point PCR	Uses primers to identify bacterial species qualitatively (not quantitatively)	Qualitative assessment of bacteria Probe for presence of specific taxa or genes (such as methicillin resistance) Low cost Rapid (<12 hr)	Not quantitative Limited by requirement for primer specificity Multiplexing is difficult
qPCR	Similar to end-point PCR, except reaction is monitored continuously to quantify the abundance of gene of interest	Quantitative analysis is possible Multiplexed (or parallel) methods reduce time and reagents required Rapid (<12 hr) Probe for presence of specific taxa or genes	Only targeted genes (amplicons) will be identified Characterization of community variation is not possible Multiplexed reactions are limited to primer sets that require similar reaction conditions
Sanger sequencing	Provides nucleotide sequence of amplicons from pure sample	Inexpensive Rapid (~24 hr) Useful for identifying cultured bacteria	Requires pure monoculture as input, so is susceptible to the same issues as traditional culturing
RNA sequencing	Same as DNA-based technique after an initial step reverse-transcribing cDNA from RNA	Informs which genetic elements are being actively transcribed, indicating biological activity Can inform bacterial viability and host response Speed similar to DNA-based techniques after ~2-hr reverse transcription step	RNA has increased sensitivity to degradation Slow (days to weeks)
NGS	Massively parallel sequencing of NAs; most commonly all variants of the 16S rRNA gene in a sample are sequenced to determine microbial species abundance	Can identify taxa in polymicrobial samples Inexpensive if many samples are run together Allows community analysis of all variants	More expensive and time-intensive than qPCR or Sanger sequencing Increased probability that background or contamination will be amplified Sensitive to contamination Database limitations Slow (4 days to 6 weeks)
Metagenomic NGS	Uses random primers to comprehensively amplify all fragments of NA sequences in a sample	Can generate information about all genes present in sample (such as identification of microbial species as well as virulence and resistance genes)	More expensive than 16S rRNA NGS Additional information can be more difficult to interpret Database limitations Slow (4 days to 6 weeks)

primer that bind to either side of the region of interest. DNA polymerase recognizes regions where primers have annealed and amplifies the DNA to create double-stranded DNA. This is repeated ≥30 times, with the DNA concentration doubling after every cycle. Once enough of the double-stranded DNA amplicon (or product of amplification events) has been produced, it can be visualized by running it on a gel (Fig. 1). Because the product of this reaction is only observed at the end of all cycles, this technique is called end-point PCR (Table I). It has been applied in studies of musculoskeletal infection and sepsis (Table II; see also Appendix Supplemental Table 1)[35,36].

Quantitative PCR (qPCR)

The qPCR methods are based on principles that are identical to those of end-point PCR[37]. However, instead of amplicon detection only at the end of all cycles, the reaction is monitored continuously at each cycle to quantitatively determine the amount of the gene of interest in the sample (Fig. 1). This also has been applied to musculoskeletal infection (see Appendix Supplemental Table 1)[38,39]. In multiplex qPCR, several PCR reactions for specific targets are performed in the same reaction mix. Results are teased apart due to differing amplicon length or release of fluorescent label upon successful amplification (Tables I and II).

Sanger Sequencing

In Sanger sequencing (Table I), the sequence of an amplicon is deduced by determining the identity of the base at each position over the amplicon length (Fig. 1)[40]. This is accomplished by including terminating nucleotides in the reaction mix, which prevent the PCR from proceeding. By measuring the length of the amplicon and knowing the identity of the succession of terminating nucleotides at each step, the identity of the base at each position can be inferred. Modern technology has allowed incorporation of fluorescent labels, instead of radioactively labeled nucleotides, that can be run on a flow cytometer and read automatically.

Sanger sequencing of the 16S rRNA gene can determine the probable identity of bacteria by comparing the determined sequence with a database of known 16S rRNA gene sequences. However, this can only be done on monocultures. Sequencing a polyclonal or impure culture results in unusable sequences. Amplification occurs, but there is too much ambiguity in the base at each position for identification. Monocultures must be grown from the specimen prior to Sanger sequencing. If the most relevant microorganism is slower-growing than others, the microbiology laboratory may only identify the first colonies that grow on plates and dispose of cultures before slower-growing strains are visible (Table II).

RNA Sequencing

All of the NA-based techniques described above use DNA. If an initial step of reverse-transcribing complementary DNA (cDNA) from RNA is added, the same techniques can detect RNA in a sample (reverse-transcription PCR [RT-PCR]).

Next-Generation Sequencing (NGS)

NGS involves massively parallel sequencing of the NAs present within a sample. PCR-generated amplicons may be separated by physical methods (i.e., binding to a chip surface) or through dilution (i.e., capillary electrophoresis). The separated amplicons are then monitored and sequenced in parallel. NGS methods include nanopore sequencing (bases identified by measuring charge fluctuation as single-stranded DNA passes through a nanopore[41]), sequencing by synthesis (modern versions of Sanger sequencing in which fluorescent labels on terminating nucleotides are removed, allowing the process to continue, after observation), and sequencing by ligation (similar to Sanger sequencing except that bases are added in 3-mers or 4-mers instead of individually) (Table III)[42,43].

Single-amplicon NGS sequences all variants of a single amplicon in a single sample. This is commonly used for microbial community taxonomic composition analysis by sequencing the 16S rRNA gene (occasionally called 16S rRNA sequencing)[32]. Metagenomic NGS uses random primers to comprehensively amplify all fragments of NA sequences (the metagenome) in a sample. Random primers are designed to bind to a broad range of genome locations and do not target specific sequences.

Researchers may use ≥2 NA-based techniques in parallel or series[32]. Commonly, analysis is performed using NGS of 16S rRNA gene amplicons, followed by end-point PCR or qPCR to confirm the presence of resistance and/or virulence genes. Resequencing a sample to identify the presence of resistance genes is more rapid than waiting for culture-based antibiotic resistance analysis.

NA-based techniques are multistep processes with multiple points at which contamination (introduction of non-sample-specific NAs) can occur (Fig. 2, Table IV), including initial collection, NA extraction, initial PCR, sequencing, and post-sequencing data processing.

Potential Benefits of Molecular Pathogen Identification Strategies

Molecular diagnostic strategies show real promise in advancing how infection is defined and how pathogens are identified for targeted treatment. Until recently, the definition of infection has been based around positive cultures. However, this excludes culture-negative infections, creating both diagnostic and treatment challenges. These issues have led to the development of diagnostic criteria incorporating the Musculoskeletal Infection Society (MSIS)[44,45] and fracture-related infection[46] consensus definitions. Several biomarkers and clinical findings have been identified to aid in the diagnosis[47-50], and these have been integrated into consensus definitions. However, although these biomarkers help to establish the presence of infection, they do not identify organisms and are, therefore, unable to guide targeted treatment. Furthermore, there appears an indeterminate subset of patients who are not mounting an aggressive inflammatory response (one resulting in signs such as purulence, a sinus tract, elevated biomarkers) who may also have clinically relevant infections, such as in the setting of nonunion or aseptic loosening of prosthetic joints. We anticipate that a thoughtful, data-driven molecular diagnostic approach may inform our overall understanding of what constitutes an infection in a treatment-oriented manner.

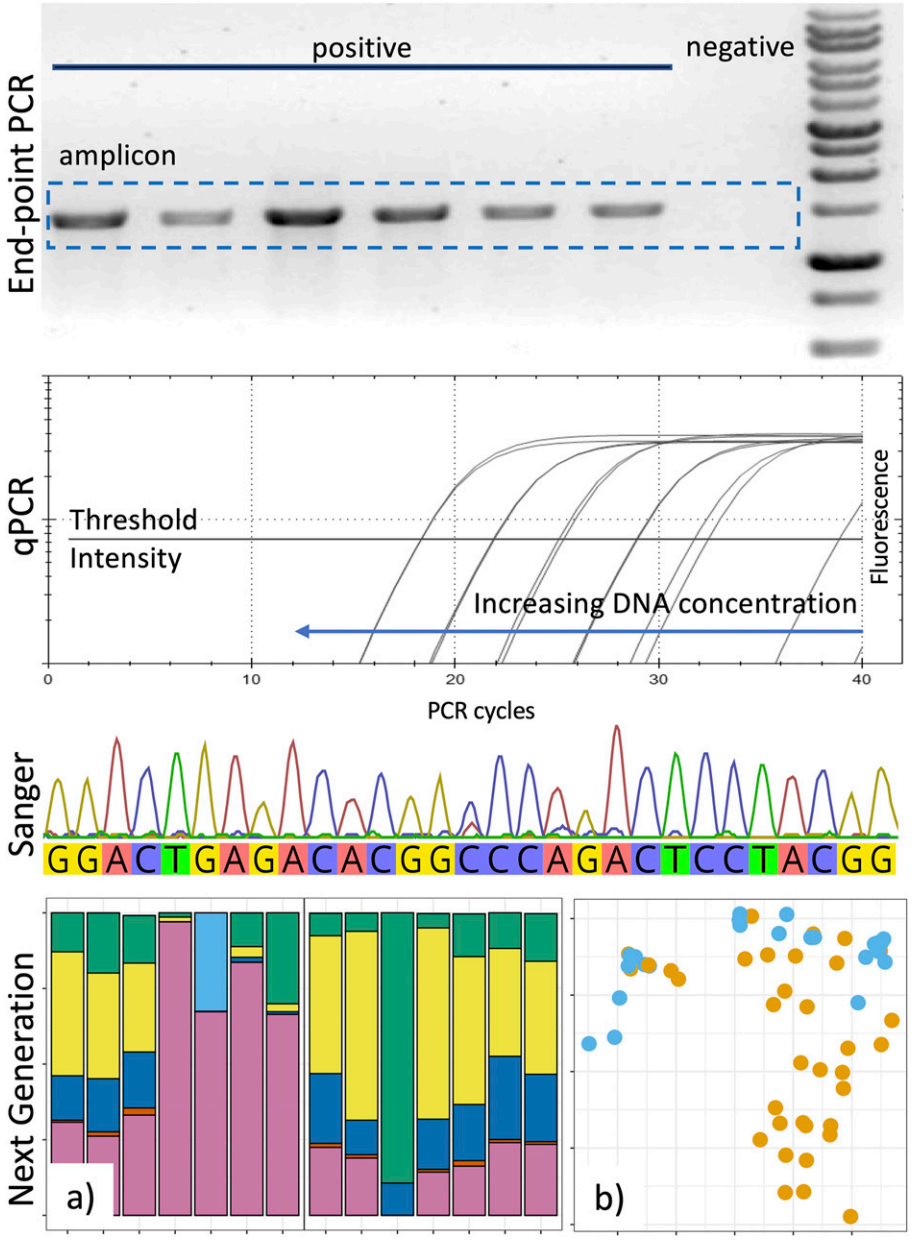

Fig. 1

Typical output of NA-based molecular techniques. In the top image, end-point PCR results are visualized as bands on an agarose gel. DNA fragments (amplicons) travel through the gel based on the number of nucleotides in the sequence (size), with shorter amplicons moving faster. When an amplicon is produced via PCR, a band can be seen. The intensity of the band indicates the concentration of the amplicon in the reaction, but the width of the band is not relevant. Reference ladder(s) containing multiple amplicons of known sizes (far right) are included on the gel for comparison. A positive result is observed as a band on the gel that has traveled the same distance as band(s) in the ladder corresponding to the size of the region of interest. No band (second from right) or a band of the wrong size indicates a negative result. In the second image, qPCR results are visualized in an amplification plot. Fluorophores are released after each successful amplification of the region of interest, resulting in an increase in fluorescence intensity (y axis) as the concentration of DNA increases in the reaction well. The fewer cycles of PCR (x axis) that a reaction must undergo to reach a threshold fluorescence (horizontal bar), the higher the initial concentration of DNA in the sample. In the third image, Sanger sequencing results are visualized as a chromatogram. Terminating fluorophores at each position in the DNA sequence are observed as peaks in fluorescence. At each position in the amplicon, the specific fluorescence (corresponding to 1 of the 4 nucleotides) indicates the base present at that position. NGS results are visualized in many ways. In the bottom image, (a) in stacked bar charts, each bar represents a single sample and each color indicates the proportional abundance of a single taxon inferred to be present in the sample, and (b) similarity in microbial taxonomic composition between samples is often visualized via principal coordinates analysis, where each dot represents a single sample and the 2-dimensional distance between dots indicates the distance between communities.

TABLE III Comparison of NGS Technologies

Chemistry	Other Names	Accuracy (Q30*)	Run Time	Total Output Data Size	Max. Read Length	Max. Reads per Run	Input Required	Max. Samples per Run	Technology Status
Pyrosequencing	Roche 454 GS-FLX Titanium (Roche)	85%	24 hr	0.7 Gb	700 bp	500,000	Not published	Not published	Discontinued
Reversible terminator chemistry	Illumina MiSeq (Illumina)	97%	55 hr	15 Gb	2 × 300 bp	25 million	ng	192	Current
	Illumina HiSeq (Illumina)	95%	2 to 6 days	150 Gb to 1 Tb	2 × 150 bp	2 to 4 billion	ng	384	Discontinued
	Illumina NextSeq (Illumina)	75%	35 hr	90 Gb	2 × 150 bp	400 million	ng	384	Current
	Illumina genome analyzer (Illumina)	98%	3 to 10 days	4 to 25 Gb	2 × 75 bp	300 million	100 ng	12	Current
	Illumina NovaSeq (Illumina)	75%	2 days	6 Tb	350 bp	20 billion	1 to 500 ng	384	Current
	Helicos Bioscience Heliscope (Helicos Biosciences)	Lower	8 days	35 Gb	100 bp	20 million	100 ng	25	Company bankrupt
Sequencing by ligation	Ion proton, Complete Genomics (Thermo Fisher Scientific)	85%	2 to 4 hr	15 Gb	200 bp	80 million	50 ng to 1 μg	384	Current
	SOLiD (Thermo Fisher Scientific)	>99%	7 to 14 days	120 Gb	100 bp	2,400	ng	96	Current
Semiconductor with sequencing	Ion Torrent (Thermo Fisher Scientific)	>99%	2 hr	10 Mb to 1 Gb	600 bp	500	ng	8	Current
Real-time sequencing	PacBio SMRT (Pacific Biosciences)	>99%	30 hr	47 Gb	25 kbp	4 million	300 ng to 1 μg	96	Current
Nanopore	Flongle (Oxford Nanopore Technologies)	Lower	16 hr	1 to 2 Gb	4 Mb	100,000	10 pg to 1 μg	96	Current
	MinION (Oxford Nanopore Technologies)	Lower	72 hr	10 to 50 Gb	4 Mb	100,000	10 pg to 1 μg	96	Current
	GridION (Oxford Nanopore Technologies)	Lower	72 hr	10 to 50 Gb	4 Mb	100,000	10 pg to 1 μg	96	Current
	PromethION (Oxford Nanopore Technologies)	Lower	72 hr	100 to 300 Gb	4 Mb	100,000	10 pg to 1 μg	96	Current

*Q30 references the sequencing quality score. When the sequencing quality reaches Q30, virtually all of the reads will be perfect without errors or ambiguities. Q30 is considered a benchmark for quality in NGS.

Other potential benefits of molecular diagnostic strategies may yield more immediate rewards. Unlike traditional cultures, which take 3 to 10 days, delaying appropriate treatment, some molecular-based strategies can be rapid, particularly if guided by information around the clinically relevant pathogens of interest. In addition to improving the time gap from debridement to appropriate antibiotic selection (potentially preventing new biofilm formation), rapid pathogen identification could facilitate targeted intraoperative treatment approaches. Furthermore, the increased sensitivity and broad nature of DNA or RNA isolation (compared with the nutritional and environmental biases associated with culture) may identify additional pathogens that are clinically relevant, either on their own or when present in combination with others. However, there remain substantial gaps that must be addressed prior to translation into the clinical space.

Caveats to NA-Based Techniques

There are important caveats to keep in mind when evaluating the use of NA-based strategies, and specific details are needed so that study methodology can be critically evaluated. Table V summarizes critical methodologic data that are needed.

Dead Cells and/or Cell-Free NAs

Bacterial DNA may be present without viable cells (extracellular DNA). Viability can be confirmed by sending information to the microbiology laboratory for growth on specialized media.

Identification of Clinically Important Pathogen Features

Common microbiota from healthy human skin sites include some genera and species identical to known pathogens. Often, the 16S rRNA gene amplicon is not sufficient to differentiate between less problematic and more pathogenic strains (such

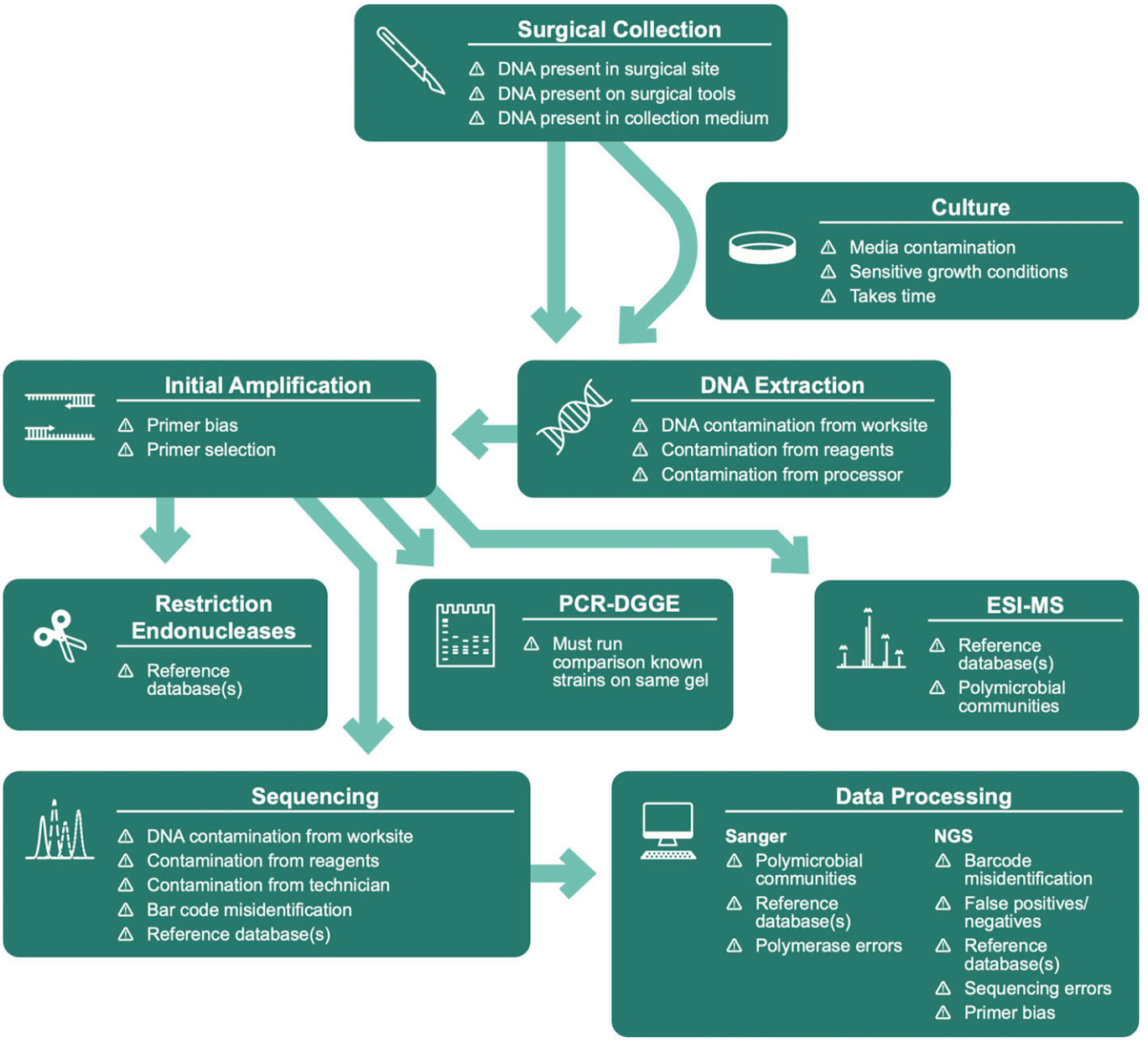

Fig. 2

Common sources of contamination and limitations or pitfalls that must be taken into account when using NA-based molecular techniques. DGGE = denaturing gradient gel electrophoresis, and ESI-MS = electrospray ionization mass spectrometry.

as methicillin-sensitive *Staphylococcus aureus* compared with methicillin-resistant *Staphylococcus aureus*). To handle this, many researchers use more than 1 NA-based technique to confirm the species or strain identity as pathogenic.

Mitochondrial Ribosomes

Mitochondrial ribosomes have sufficient similarity to bacterial ribosomes that primers designed to target bacteria will occasionally also amplify mitochondrial DNA. This can be particularly problematic in human samples, where human DNA vastly outnumbers microbial DNA.

Issues with Primers

Although 16S rRNA gene primers have been developed since the 1990s, no primer set is perfect. They are each known to have biases in which some taxa are more readily amplified than others. Potential primer biases must be considered when comparing data between experiments using different primer sets. Today, 16S rRNA gene-targeting primers designed for use with NGS applications have been tested to work well with most known clinical isolates. If using sequencing data to identify a novel pathogen, however, it is possible that primers may not be as efficient in amplifying its taxon[51]. When taxonomic identification

TABLE IV Information Necessary to Evaluate and Interpret Microbial Data Analysis*

Include with Analysis	Example(s)	Benefit	Problems if Absent
Extraction method	Kit-based In-house Automated	Easy comparison when same extraction methods are used	Extraction methods are optimized for different microbes. Harsher lysis techniques that may be necessary for spore-forming bacteria or fungi may be too harsh and degrade NAs from other microbes.
Positive extraction control	Standard microbial community	Confirms successful NA extraction	Low NA concentration may indicate failed extraction but be interpreted as low or no NA present.
Negative extraction control	Molecular biology grade water	Identifies contamination during extraction	High NA concentration may indicate contamination but be interpreted as sample with high NA abundance.
Positive PCR control	Standard DNA community	Confirms successful PCR amplification	No amplification may indicate failed reaction but be interpreted as no target present.
Negative PCR control	Molecular biology grade water	Identifies contamination of PCR reaction	Positive PCR reactions may indicate contamination but be interpreted as target present.
PCR reaction conditions	Salt concentrations Primer concentrations Enzyme brand name Thermocycling conditions	Reproducible amplification	Primer binding and enzyme efficacy can be susceptible to slight changes in reaction conditions. Future studies may fail if exact reaction conditions are not duplicated.
Primer names and sequences	Exact nucleotide sequences listed	Allows others to reproduce results in future samples	Results from studies targeting the same gene but with different primers may yield different conclusions based on primer specificity rather than biological differences.
Sequencing technology	Company and hardware and software version(s)	Different sequencing technologies have advantages and disadvantages (Table II), and results do slightly vary between technologies	Results from discontinued technologies may not be comparable with those from modern technologies.
Methods for reducing contamination	DNA extraction and post-PCR processing occurring in isolated areas	Assures reader that efforts have been made to minimize contamination	Reader may question if contamination occurred between samples.
Code for processing	GitHub repository	Reproducible analysis	Variation between data analysis may mask true variation in biological data or may falsely infer variations.
Deidentified raw data	.fasta files	Comparison with results from future studies	Nonreproducible results. Future studies must reproduce all sample types for direct comparison.
Define cutoffs or limit of detection thresholds	Minimum no. of reads to determine presence in a sample	Defines rare biosphere and the stringency of the study to account for false-positives or negatives	Low-abundance targets may be identified in some studies with low limits of detection while those with higher thresholds will miss them.
No. of reads (NGS)	Median reads per sample Variation in reads per sample	Too few reads may lead to false conclusion of microbe absence	Readers unable to determine depth of sequencing and validity of comparing rare biosphere between studies.
Normalization method for no. of reads (NGS)	Log_2 transformation Rarefaction	Standardize no. of reads per sample	Normalization methods may skew results; these skews may not be identified until future methods develop. Acknowledging the normalization method used will help future researchers to understand if they need to reprocess the data with new normalization techniques.
Define contaminants	Cutoff limits Patterns of abundance	Reproducible results	Contaminants may be identified as diagnostically important.

*When reading scientific literature, it is important to note if these items have been included. If information is not included in the research, the reader must acknowledge the problems that this absence may indicate. Sometimes the problem is merely an inability to compare with other literature, but other times, it may mean that the reported results should not be trusted until reproduced by other researchers.

TABLE V Grades of Recommendation*

Statement	Grade of Recommendation†
Positive and negative controls must be included	A
No. of reads per sample must be reported and any normalization method(s) described	B
Code used for data analysis should be publicly available	B
When comparing studies, the primers or targeted regions should be the same	B
Sequencing-based technology should be consistent when comparing studies	B
Ensure that the sequencing-based technology is currently maintained	I
Cutoff or limit of detection thresholds must be stated	B
Black-box methods should not be used	C
Publicly available, curated reference database(s) should be consulted	B

*Recommendations are based on the best evidence to date. †According to Wright[60], grade A indicates good evidence (Level-I studies with consistent findings) for or against recommending intervention; grade B, fair evidence (Level-II or III studies with consistent findings) for or against recommending intervention; grade C, poor-quality evidence (Level-IV or V studies with consistent findings) for or against recommending intervention; and grade I, insufficient or conflicting evidence not allowing a recommendation for or against intervention.

of uncultured bacteria to the species level is necessary, the 16S rRNA gene is sometimes insufficient. For example, Escherichia and Shigella cannot be differentiated by their 16S rRNA gene sequences alone. Taxonomic identification of bacteria via the 16S rRNA gene is dependent on comparing amplified sequences with a database of known sequences. Many public databases exist, each with different strengths and weaknesses in accuracy, coverage, taxonomic depth, and nomenclature.

Targeting Resistance and/or Virulence Genes

There are specific challenges associated with identifying resistance and/or virulence genes. Horizontal gene transfer spreads genes between phylogenetically distant bacteria. It is possible that simple amplification will detect genes of interest that are present in a specimen but not in the pathogenically relevant species. Naturally occurring mutations within the targeted primer-binding sites may also yield false-negatives. Additionally, for almost every mode of antibiotic resistance, there exist multiple responsible genes. It is not possible to design primers that will universally detect all resistance and/or virulence genes or even that will detect the same gene in all taxa. In cases where identification of a broad range of antibiotic resistance genes is necessary, metagenomic analyses are recommended over single-gene-targeted PCR.

Analysis of Complex Data

A new complexity for clinicians to consider is the large amount of data yielded from a single sample. These data may include community surveys of variation in a single gene (i.e., the

TABLE VI Specimen Storage Solutions and Their Suitability for Downstream Applications*

Storage Solution	Culture	Culture After Freezing	DNA	RNA
None[61]	+	—	+	—
Saline solution	++	—	+	—
Nutrient broth	++	—	—	—
Amies transport medium[62]	+++	+	++	—
15% glycerol[63-65]	+++	+++	++	—
Lysis buffer (i.e., Longmire)[63,64]	—	—	++	+
NA-stabilization solution (i.e., RNAlater[66])	—	—	+	+++
Phenol (i.e., TRIzol)[67]	—	—	++	+++
95% ethanol[68]	—	—	+	—
Formaldehyde or formalin[69,70]	—	—	+	—

*— = not recommended, + = possible but not ideal, ++ = good, and +++ = recommended for best results.

TABLE VII Clinical Utility of Each NA-Based Analysis Technique

Technique	Clinical Utility
End-point PCR	Good basic technique that will likely maintain utility
	Most useful for identification of specific targeted taxa and genes
	Adaptable for rapid point-of-care testing in the operating room
qPCR	Widely used in other clinical settings (e.g., SARS-CoV-2 testing)
	Useful to detect taxa or genes without first isolating bacterial cultures
	Adaptable for rapid point-of-care testing in the operating room
Sanger sequencing	Excellent technique for classifying or categorizing cultured microbes that cannot be identified using culture-based patterns
	Likely minimal clinical utility
	Rapid but dependent on first isolating pure culture
RNA sequencing	Currently used only in research; however, future clinical application may target identification of transcriptionally active bacteria
Amplicon-targeted NGS	Currently used primarily for research
	Can be considered for recalcitrant infection or when cultures are presumed to be inadequate (such as culture-negative infection); in this setting, results must be interpreted with extreme caution
	Potential for future clinical use as part of standard of care once issues have been addressed; some issues that must be addressed include, but are not limited to:
	• Shortening data generation and analysis time
	• Establishing "read" thresholds separating positive results from potential contaminants
	• Identifying pathogenic compared with non-pathogenic species
Metagenomic NGS	For research applications currently, but variations may be clinically relevant in future
	Future utility likely in identification of virulence or resistance genes present in infecting microorganisms; issues similar to those of amplicon NGS must be addressed prior to advancing into clinical practice

bacterial 16S rDNA gene) or broad community analysis of randomly amplified regions (i.e., metagenomic sequencing). The large amount of data produced by NGS necessitates more complicated data processing post-sequencing. This processing includes binding small sequences together (forming paired-end reads, scaffolds, and contigs) as well as comparing output sequences with existing databases (taxonomy assignment, scaffold testing, gene annotation)[52]. Based on this complexity, clinicians should consider caution when considering whether to use companies that market an ability to convert raw data to diagnostic results without offering insights into methods and protocols (black-box methods).

NGS

NGS is a powerful tool with incredible sensitivity that can hypothetically detect a single copy of a gene in 10 μL of a sample. Because such a small starting mass may yield a positive result, false-positives are a known confounding factor, particularly in samples with a low input mass (Fig. 2). There are several methods that can minimize this risk.

Each NGS technology has benefits and problems (Table IV). No single technology can concomitantly provide long amplicons, accurate reads, large numbers of reads, fast run time, and low cost using small sample inputs. Researchers must choose which of these components are most important to their application and must also consider whether the extra information received from NGS technology is worth the extra time, cost, and potential for a confounding diagnosis from detected, but not necessarily clinically relevant, pathogens.

Diagnosis based solely on NGS results is not currently recommended because of the risk of overdiagnosis (identifying the presence of bacterial taxa without confirming viability and/or pathogenicity) and subsequent overtreatment. Not enough studies have been performed to understand whether NGS can be used as a stand-alone diagnostic tool and how results should be interpreted. With that caveat acknowledged, when dealing with infections that have failed to respond to standard treatments, NGS may help to elucidate the presence of uncommon or previously undetected pathogens.

Best Practices for Collecting Specimens

Specimen collection and storage can affect NA-based diagnostic protocols. Solutions used in surgical treatment, especially antiseptics and disinfectants, may degrade NAs or inhibit enzymes. For this reason, specimens should be collected prior to any treatment. If collecting samples from multiple sites, it is important to ensure that no cross-contamination occurs. Ideally, specimens will pass directly into the collection medium. If intermediate surfaces are unavoidable, NA-free, or PCR-clean, supplies should be used. Standard materials may be rendered

TABLE VIII Suggested Negative Controls, Positive Controls, and Contaminant-Source Identification When Preparing Samples for NGS

	Negative	Positive	Contamination Source(s)
Collection	Sterile storage solution	Not commonly performed	Patient skin flora
			Irrigation fluid
			Instruments
			Clinician
Extraction	Reagents	Microbial community standard	Technician
			Reagents
			Environment
			Parallel samples
PCR	Water-only	Microbial community NA standard	Technician
			Reagents
			Environment
			Parallel samples
Sequencing	No PCR water	Successful PCR amplification	Technician
			Reagents
			Parallel samples
Processing	Empty primer indices	Previously processed data	Improper analysis
			Comparison database

PCR-clean via either treatment with RNase AWAY (Thermo Fisher Scientific) or bleach followed by rinsing with molecular biology-grade water[53] or treatment with autoclaving on an extended steam cycle of ≥80 minutes[54]. As discussed in the caveats section, even sterile items such as surgical drapes or gloves may harbor trace amounts of NAs[55] that will not harm patients but may contaminate specimens. The specific application used for analysis will influence the best collection medium and storage conditions (Table VI). If >1 type of test is to be performed on a specimen, it is usually better to take multiple samples from the same site and treat each independently.

Best Practices for Clinical Use

Although there is real potential for culture-independent diagnostic strategies to improve our diagnostic capacity by improving sensitivity and identifying microbial species that may be missed using traditional culture, these strategies are not yet ready for regular use as part of standard-of-care practice for several reasons. First, more carefully controlled microbiome-focused orthopaedic wound research must be performed to address outstanding questions with regard to the relationship between sensitivity and reproducibility in the detection of wound-associated microbes. This may necessitate closer collaborative relationships between orthopaedic surgeons and microbiome researchers, in lieu of commercial black-box microbiome sequencing providers. Second, there are important technical and logistical hurdles involving the infrastructure needed for the analysis of NA-based diagnostic tools, particularly NGS. Furthermore, the time required from the operating room to the data analysis and robust interpretation needed to inform clinical decision-making is currently impractically long. To date, most orthopaedic research applying NA-based techniques has been in arthroplasty, with a limited number of studies in trauma and orthopaedics more generally, and NGS is becoming increasingly important in these studies (see Appendix Supplemental Table 1). Newer advances in sequencing technology, particularly from Oxford Nanopore Technologies, and the rapid increase and spread of bioinformatics training among the biomedical workforce make the clinical use of NGS techniques in orthopaedic settings an optimistic goal for the coming years. Table VII outlines the clinical utility anticipated for these NA-based techniques.

Best Practices for Interpretation of These Complex Data Sets

There are several important metrics to keep in mind when evaluating research using NA-based technology. There are clear tradeoffs among speed, accuracy, sensitivity, price per sample, and coverage. Investigators and clinicians must carefully consider tradeoffs when selecting sequencing methods.

When evaluating published research and comparing results, it is important to keep the following in mind (Tables V and VIII):

1. Were appropriate positive and negative controls included at each step, and are these results reported? NGS studies should include positive controls sequencing communities of known composition and negative controls that sequence samples where no community is expected (Table VIII).
2. Is the number of reads per sample reported?

3. Is the code used for data analysis publicly available for other researchers to examine?
4. Are primers and/or targeted regions the same between compared studies? Both the efficacy and sensitivity of primers can be different during amplification and when comparing sequences with existing databases[56,57]. The ease of amplification of microbial groups changes with changes in primers, salt concentrations, temperatures, and other variables.
5. Is the sequencing technology consistent between studies? If not, how do biases of different technologies affect the results?
6. Is the technology currently maintained? Technologies present in published literature for only a short period of time must be treated with skepticism.
7. The cutoff or limit of detection thresholds should be stated along with definitions of contaminants. Whenever possible, deidentified raw data should be publicly available so that another researcher may repeat the analysis or compare the raw reads with those from samples produced in future studies.
8. Achieving NGS data with the exact same number of reads per sample is impossible and the read counts can vary quite a bit between specimens[58]. In experiments containing an uneven number of reads per sample (a >10-fold difference), the researcher must consider resequencing outlier samples or normalize the data to compare samples more accurately using strategies such as rarefication.
9. Methods sections of published papers should include description of methods applied to reduce false-positives, such as experimental controls to reduce the identification of false-positives, well-defined threshold of reads per sample (≥2,000)[59], removal of taxa present in samples in only 1 or 2 reads, and removal of taxa whose abundance is linearly related to the volume of the samples analyzed.

Conclusions

Molecular diagnostic strategies will become increasingly important in the diagnosis of infection and identification of pathogens, both in research and in clinical practice. However, for these techniques to be effectively applied to orthopaedics, clinicians and clinician-scientists must better understand the nuances, appropriate applications, and the limitations associated with each of these assessment tools. We anticipate that this review may provide a mechanism for generating hypotheses, improving standards, designing better studies, and enhancing our ability to effectively interpret and apply published research.

Appendix

eA Supporting material provided by the authors is posted with the online version of this article as a data supplement at jbjs.org (http://links.lww.com/JBJS/H421). ■

Emily Ann McClure, PhD[1]
Paul Werth, PhD[2]
Benjamin Ross, PhD[1]
Ida Leah Gitajn, MD, MS[2]

[1]Dartmouth College, Hanover, New Hampshire

[2]Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire

Email for corresponding author: Ida.Leah.Gitajn@hitchcock.org

References

1. Horton SA, Hoyt BW, Zaidi SMR, Schloss MG, Joshi M, Carlini AR, Castillo RC, O'Toole RV. Risk factors for treatment failure of fracture-related infections. Injury. 2021 Jun;52(6):1351-5.

2. Obremskey WT, Schmidt AH, O'Toole RV, DeSanto J, Morshed S, Tornetta P III, Murray CK, Jones CB, Scharfstein DO, Taylor TJ, Carlini AR, Castillo RC, Morshed S; METRC. A prospective randomized trial to assess oral versus intravenous antibiotics for the treatment of postoperative wound infection after extremity fractures (POvIV Study). J Orthop Trauma. 2017 Apr;31 Suppl 1:S32-S38.

3. Bosse MJ, MacKenzie EJ, Kellam JF, Burgess AR, Webb LX, Swiontkowski MF, Sanders RW, Jones AL, McAndrew MP, Patterson BM, McCarthy ML, Travison TG, Castillo RC. An analysis of outcomes of reconstruction or amputation after leg-threatening injuries. N Engl J Med. 2002 Dec 12;347(24):1924-31.

4. Huh J, Stinner DJ, Burns TC, Hsu JR; Late Amputation Study Team. Infectious complications and soft tissue injury contribute to late amputation after severe lower extremity trauma. J Trauma. 2011 Jul;71(1 Suppl):S47-51.

5. Melcer T, Sechriest VF, Walker J, Galarneau M. A comparison of health outcomes for combat amputee and limb salvage patients injured in Iraq and Afghanistan wars. J Trauma Acute Care Surg. 2013 Aug;75(2)(Suppl 2):S247-54.

6. Zmistowski B, Tetreault MW, Alijanipour P, Chen AF, Della Valle CJ, Parvizi J. Recurrent periprosthetic joint infection: persistent or new infection? J Arthroplasty. 2013 Oct;28(9):1486-9.

7. Shahi A, Tan TL, Chen AF, Maltenfort MG, Parvizi J. In-hospital mortality in patients with periprosthetic joint infection. J Arthroplasty. 2017 Mar;32(3):948-952.e1.

8. Parvizi J, Pawasarat IM, Azzam KA, Joshi A, Hansen EN, Bozic KJ. Periprosthetic joint infection: the economic impact of methicillin-resistant infections. J Arthroplasty. 2010 Sep;25(6)(Suppl):103-7.

9. Bozic KJ, Lau E, Kurtz S, Ong K, Berry DJ. Patient-related risk factors for post-operative mortality and periprosthetic joint infection in Medicare patients undergoing TKA. Clin Orthop Relat Res. 2012 Jan;470(1):130-7.

10. Kurtz SM, Lau EC, Son MS, Chang ET, Zimmerli W, Parvizi J. Are we winning or losing the battle with periprosthetic joint infection: trends in periprosthetic joint infection and mortality risk for the Medicare population. J Arthroplasty. 2018 Oct;33(10):3238-45.

11. Bozic KJ, Ries MD. The impact of infection after total hip arthroplasty on hospital and surgeon resource utilization. J Bone Joint Surg Am. 2005 Aug;87(8):1746-51.

12. Tan TL, Kheir MM, Shohat N, Tan DD, Kheir M, Chen C, Parvizi J. Culture-negative periprosthetic joint infection: an update on what to expect. JB JS Open Access. 2018 Jul 12;3(3):e0060.

13. Parvizi J, Erkocak OF, Della Valle CJ. Culture-negative periprosthetic joint infection. J Bone Joint Surg Am. 2014 Mar 5;96(5):430-6.

14. Berbari EF, Marculescu C, Sia I, Lahr BD, Hanssen AD, Steckelberg JM, Gullerud R, Osmon DR. Culture-negative prosthetic joint infection. Clin Infect Dis. 2007 Nov 1;45(9):1113-9.

15. Font-Vizcarra L, García S, Bori G, Martinez-Pastor JC, Zumbado A, Morata L, Mensa J, Soriano A. Long-term results of acute prosthetic joint infection treated with debridement and prosthesis retention: a case-control study. Int J Artif Organs. 2012 Oct;35(10):908-12.

16. Tarabichi M, Shohat N, Goswami K, Alvand A, Silibovsky R, Belden K, Parvizi J. Diagnosis of periprosthetic joint infection: the potential of next-generation sequencing. J Bone Joint Surg Am. 2018 Jan 17;100(2):147-54.

17. Gitajn IL, Sprague S, Petrisor BA, Jeray KJ, O'Hara NN, Nascone JW, Bhandari M, Slobogean GP. Predictors of complications in severe open fractures. Read at the Annual Meeting of the Orthopaedic Trauma Association; 2017 Oct 14. Paper no. 128.

18. Lewis WH, Tahon G, Geesink P, Sousa DZ, Ettema TJG. Innovations to culturing the uncultured microbial majority. Nat Rev Microbiol. 2021 Apr;19(4):225-40.

19. Rajilić-Stojanović M, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. Environ Microbiol. 2007 Sep;9(9):2125-36.

20. Misic AM, Gardner SE, Grice EA. The wound microbiome: modern approaches to examining the role of microorganisms in impaired chronic wound healing. Adv Wound Care (New Rochelle). 2014 Jul 1;3(7):502-10.

21. Palmer MP, Altman DT, Altman GT, Sewecke JJ, Ehrlich GD, Hu FZ, Nistico L, Melton-Kreft R, Gause TM 3rd, Costerton JW. Can we trust intraoperative culture results in nonunions? J Orthop Trauma. 2014 Jul;28(7):384-90.

22. Bartow-McKenney C, Hannigan GD, Horwinski J, Hesketh P, Horan AD, Mehta S, Grice EA. The microbiota of traumatic, open fracture wounds is associated with mechanism of injury. Wound Repair Regen. 2018 Mar;26(2):127-35.

23. Ibberson CB, Whiteley M. The social life of microbes in chronic infection. Curr Opin Microbiol. 2020 Feb;53:44-50.

24. Nguyen AT, Oglesby-Sherrouse AG. Interactions between Pseudomonas aeruginosa and Staphylococcus aureus during co-cultivations and polymicrobial infections. Appl Microbiol Biotechnol. 2016 Jul;100(14):6141-8.

25. Limoli DH, Yang J, Khansaheb MK, Helfman B, Peng L, Stecenko AA, Goldberg JB. Staphylococcus aureus and Pseudomonas aeruginosa co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. Eur J Clin Microbiol Infect Dis. 2016 Jun;35(6):947-53.

26. Hotterbeekx A, Kumar-Singh S, Goossens H, Malhotra-Kumar S. *In vivo* and *in vitro* interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. Front Cell Infect Microbiol. 2017 Apr 3;7:106.

27. Peleg AY, Hogan DA, Mylonakis E. Medically important bacterial-fungal interactions. Nat Rev Microbiol. 2010 May;8(5):340-9.

28. Orazi G, O'Toole GA. *Pseudomonas aeruginosa* alters Staphylococcus *aureus* sensitivity to vancomycin in a biofilm model of cystic fibrosis infection. mBio. 2017 Jul 18;8(4):e00873-17.

29. Goswami K, Parvizi J. Culture-negative periprosthetic joint infection: is there a diagnostic role for next-generation sequencing? Expert Rev Mol Diagn. 2020 Mar; 20(3):269-72.

30. Tang Y, Zhao D, Wang S, Yi Q, Xia Y, Geng B. Diagnostic value of next-generation sequencing in periprosthetic joint infection: a systematic review. Orthop Surg. 2022 Feb;14(2):190-8.

31. Woese CR, Fox GE. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci U S A. 1977 Nov;74(11):5088-90.

32. Hugenholtz P, Chuvochina M, Oren A, Parks DH, Soo RM. Prokaryotic taxonomy and nomenclature in the age of big sequence data. ISME J. 2021 Jul;15(7):1879-92.

33. Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol. 2009;2009:574398.

34. Mullis KB, Faloona FA. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol. 1987;155:335-50.

35. Blaschke AJ, Heyrend C, Byington CL, Fisher MA, Barker E, Garrone NF, Thatcher SA, Pavia AT, Barney T, Alger GD, Daly JA, Ririe KM, Ota I, Poritz MA. Rapid identification of pathogens from positive blood cultures by multiplex polymerase chain reaction using the FilmArray system. Diagn Microbiol Infect Dis. 2012 Dec;74(4):349-55.

36. Wood JB, Sesler C, Stalons D, Grigorenko E, Schoenecker JG, Creech CB, Thomsen IP. Performance of TEM-PCR vs culture for bacterial identification in pediatric musculoskeletal infections. Open Forum Infect Dis. 2018 May 22;5(6):ofy119.

37. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 2019 Jul;37(7):761-74.

38. Fang XY, Li WB, Zhang CF, Huang ZD, Zeng HY, Dong Z, Zhang WM. Detecting the presence of bacterial DNA and RNA by polymerase chain reaction to diagnose suspected periprosthetic joint infection after antibiotic therapy. Orthop Surg. 2018 Feb;10(1):40-6.

39. Berns E, Barrett C, Gardezi M, Spake C, Glasser J, Antoci V, Born CT, Garcia DR. Current clinical methods for detection of peri-prosthetic joint infection. Surg Infect (Larchmt). 2020 Oct;21(8):645-53.

40. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463-7.

41. Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. Nat Biotechnol. 2021 Nov;39(11):1348-65.

42. Stark R, Grzelak M, Hadfield J. RNA sequencing: the teenage years. Nat Rev Genet. 2019 Nov;20(11):631-56.

43. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. Trends Genet. 2014 Sep;30(9):418-26.

44. Parvizi J, Zmistowski B, Berbari EF, Bauer TW, Springer BD, Della Valle CJ, Garvin KL, Mont MA, Wongworawat MD, Zalavras CG. New definition for periprosthetic joint infection: from the Workgroup of the Musculoskeletal Infection Society. Clin Orthop Relat Res. 2011 Nov;469(11):2992-4.

45. Parvizi J, Tan TL, Goswami K, Higuera C, Della Valle C, Chen AF, Shohat N. The 2018 Definition of Periprosthetic Hip and Knee Infection: an evidence-based and validated criteria. J Arthroplasty. 2018 May;33(5):1309-1314.e2.

46. Metsemakers WJ, Morgenstern M, McNally MA, Moriarty TF, McFadyen I, Scarborough M, Athanasou NA, Ochsner PE, Kuehl R, Raschke M, Borens O, Xie Z, Velkes S, Hungerer S, Kates SL, Zalavras C, Giannoudis PV, Richards RG, Verhofstad MHJ. Fracture-related infection: a consensus on definition from an international expert group. Injury. 2018 Mar;49(3):505-10.

47. Wyatt MC, Beswick AD, Kunutsor SK, Wilson MJ, Whitehouse MR, Blom AW. The alpha-defensin immunoassay and leukocyte esterase colorimetric strip test for the diagnosis of periprosthetic infection: a systematic review and meta-analysis. J Bone Joint Surg Am. 2016 Jun 15;98(12):992-1000.

48. Aggarwal VK, Tischler E, Ghanem E, Parvizi J. Leukocyte esterase from synovial fluid aspirate: a technical note. J Arthroplasty. 2013 Jan;28(1):193-5.

49. Gehrke T, Lausmann C, Citak M, Bonanzinga T, Frommelt L, Zahar A. The accuracy of the alpha defensin lateral flow device for diagnosis of periprosthetic joint infection: comparison with a gold standard. J Bone Joint Surg Am. 2018 Jan 3;100(1):42-8.

50. Yoon JR, Yang SH, Shin YS. Diagnostic accuracy of interleukin-6 and procalcitonin in patients with periprosthetic joint infection: a systematic review and meta-analysis. Int Orthop. 2018 Jun;42(6):1213-26.

51. Frank JA, Reich CI, Sharma S, Weisbaum JS, Wilson BA, Olsen GJ. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol. 2008 Apr;74(8):2461-70.

52. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, Perry T, Kao D, Mason AL, Madsen KL, Wong GK. Characterization of the gut microbiome using 16S or shotgun metagenomics. Front Microbiol. 2016 Apr 20;7:459.

53. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A. 2011 Mar 15;108(Suppl 1)(Suppl 1):4516-22.

54. Nilsson M, De Maeyer H, Allen M. Evaluation of different cleaning strategies for removal of contaminating DNA molecules. Genes (Basel). 2022 Jan 17;13(1):162.

55. Suyama T, Kawaharasaki M. Decomposition of waste DNA with extended autoclaving under unsaturated steam. Biotechniques. 2013 Dec;55(6):296-9.

56. Taranger J, Trollfors B, Lind L, Zackrisson G, Beling-Holmquist K. Environmental contamination leading to false-positive polymerase chain reaction for pertussis. Pediatr Infect Dis J. 1994 Oct;13(10):936-7.

57. Kumar PS, Brooker MR, Dowd SE, Camerlengo T. Target region selection is a critical determinant of community fingerprints generated by 16S pyrosequencing. PLoS One. 2011;6(6):e20956.

58. Nearing JT, Comeau AM, Langille MGI. Identifying biases and their potential solutions in human microbiome studies. Microbiome. 2021 May 18;9(1):113.

59. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER, Knight R. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome. 2017 Mar 3;5(1):27.

60. Wright JG. Revised grades of recommendation for summaries or reviews of orthopaedic surgical studies. J Bone Joint Surg Am. 2006 May;88(5):1161-2.

61. Mutter GL, Zahrieh D, Liu C, Neuberg D, Finkelstein D, Baker HE, Warrington JA. Comparison of frozen and RNALater solid tissue storage methods for use in RNA expression microarrays. BMC Genomics. 2004 Nov 10;5:88.

62. Wiehlmann L, Pienkowska K, Hedtfeld S, Dorda M, Tümmler B. Impact of sample processing on human airways microbial metagenomes. J Biotechnol. 2017 May 20;250:51-5.

63. Camacho-Sanchez M, Burraco P, Gomez-Mestre I, Leonard JA. Preservation of RNA and DNA from mammal samples under field conditions. Mol Ecol Resour. 2013 Jul;13(4):663-73.

64. Deschamps C, Fournier E, Uriot O, Lajoie F, Verdier C, Comtet-Marre S, Thomas M, Kapel N, Cherbuy C, Alric M, Almeida M, Etienne-Mesmin L, Blanquet-Diot S. Comparative methods for fecal sample storage to preserve gut microbial structure and function in an in vitro model of the human colon. Appl Microbiol Biotechnol. 2020 Dec;104(23):10233-47.

65. Claassen-Weitz S, Gardner-Lubbe S, Mwaikono KS, du Toit E, Zar HJ, Nicol MP. Optimizing 16S rRNA gene profile analysis from low biomass nasopharyngeal and induced sputum specimens. BMC Microbiol. 2020 May 12;20(1):113.

66. Wu Z, Hullings AG, Ghanbari R, Etemadi A, Wan Y, Zhu B, Poustchi H, Fahraji BB, Sakhvidi MJZ, Shi J, Knight R, Malekzadeh R, Sinha R, Vogtmann E. Comparison of fecal and oral collection methods for studies of the human microbiota in two Iranian cohorts. BMC Microbiol. 2021 Nov 22;21(1):324.

67. Alabi T, Patel SB, Bhatia S, Wolfson JA, Singh P. Isolation of DNA-free RNA from human bone marrow mononuclear cells: comparison of laboratory methods. Biotechniques. 2020 Mar;68(3):159-62.

68. Ayana M, Cools P, Mekonnen Z, Biruksew A, Dana D, Rashwan N, Prichard R, Vlaminck J, Verweij JJ, Levecke B. Comparison of four DNA extraction and three preservation protocols for the molecular detection and quantification of soil-transmitted helminths in stool. PLoS Negl Trop Dis. 2019 Oct 28;13(10):e0007778.

69. Einaga N, Yoshida A, Noda H, Suemitsu M, Nakayama Y, Sakurada A, Kawaji Y, Yamaguchi H, Sasaki Y, Tokino T, Esumi M. Assessment of the quality of DNA from various formalin-fixed paraffin-embedded (FFPE) tissues and the use of this DNA for next-generation sequencing (NGS) with no artifactual mutation. PLoS One. 2017 May 12;12(5):e0176280.

70. Hykin SM, Bi K, McGuire JA. Fixing formalin: a method to recover genomic-scale DNA sequence data from formalin-fixed museum specimens using high-throughput sequencing. PLoS One. 2015 Oct 27;10(10):e0141579.