PCR Is Changing Clinical Diagnostics

PCR outperforms traditional culture-based diagnostic procedures that often miss bacteria when they fail to grow.

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PCR combined with mass spectrometry is providing a whole new way to identify bacteria, particularly clinically important pathogens. For years, unculturable bacteria troubled physicians observing signs in their patients that were characteristic of infections while reading “no growth” on lab reports. Indeed, culture-based microbiological techniques are severely limited in their ability to detect pathogens. Culture-negative infections result primarily from bacteria adopting the biofilm mode of growth or responding to bacteriostatic antibiotics.

The failure to culture pathogenic bacteria reflects our inability to replicate the environment in which they grow. Inappropriate media, missing chemical signals, and the buildup of toxic waste products can contribute to the bacteriology lab’s inability to culture bacteria. Moreover, current techniques favor particular groups of cultivable planktonic bacteria, although only a very few bacteria exist in planktonic isolation. Instead they more often live harmoniously with other bacteria in complex biofilms. Most naturally occurring biofilms are polymicrobial communities, held together with bacterially and host-produced extracellular polymeric substances. The biofilm structure protects the bacteria from external forces, including the host immune system. Thus, biofilms provide challenges in terms of diagnoses and treatments.

PCR Provides Alternative to Culture-Based Detection

PCR findings confirm that traditional culture-based analyses miss many bacteria that fail to grow readily. The sensitivity provided by these nucleic acid amplification technologies continues to advance our understanding of complex microbial communities. However, despite their great specificity, early PCR techniques were not suitable for routine use by the clinical diagnostic community due to the inability to effectively implement them on a large scale.

A great advantage of PCR is specificity. Users can target DNA with specific primers, out-performing traditional culture methods. However, specificity can prove a disadvantage when a PCR reaction targets only one or a small group of highly related species. Thus, first generation PCR diagnostic methods suffered because of the large number of reactions that were required to provide comprehensive coverage for possible pathogenic species. Additionally, highly trained personnel were needed to conduct first generation diagnostic assays—for example, to ensure carry-over-free PCR during day-to-day operations. To be workable, diagnostic assays need to be easy, accurate, inexpensive, and comprehensive. They must also clarify, not complicate, the clinical decision process.

While the first-generation PCR diagnostic procedures failed, the second-generation technologies excel by enabling massively parallel testing of organisms. Results from parallel testing of organisms are compared electronically to more than 750,000 standards.

Because PCR can yield false positives, secondary confirmation is recommended, including via 16S fluorescence in situ hybridization and deep 16S sequencing.

DNA-based technologies consistently support one another and outperform culture-based tests, particularly when used to detect polymicrobial infections.

These diagnostic technologies are adept when dealing with orthopedic, chronic wound, and hospital acquired infections.

SUMMARY

➤ PCR provides a new way to identify bacteria, particularly clinically important pathogens that prove unculturable via standard diagnostic protocols. However, first-generation PCR diagnostic methods suffered because skilled personnel were required to carry out large numbers of reactions.

➤ PCR is now combined with mass spectrometry and fully automated; results from parallel testing of organisms are compared electronically to more than 750,000 standards.

➤ Because PCR can yield false positives, secondary confirmation is recommended, including via 16S fluorescence in situ hybridization and deep 16S sequencing.

➤ DNA-based technologies consistently support one another and outperform culture-based tests, particularly when used to detect polymicrobial infections.

➤ These diagnostic technologies are adept when dealing with orthopedic, chronic wound, and hospital acquired infections.
testing of organisms and improving workflow and throughput. These second-generation systems use primers that target conserved eubacterial sequences and then use mass spectrometry (MS) to analyze the resulting amplimers on the basis of their molecular weights. The molecular weight of each amplimer is then uniquely deconvoluted to determine its exact base composition from which the bacterial species is unambiguously determined by comparison with a comprehensive sequence composition database. Readout of the infecting species is facilitated by user-friendly automated interfaces.

**Changing Approaches to Clinical Diagnostics**

This technology is poised to change the landscape of clinical microbiology laboratories. One of these technologies is being commercialized by Ibis Biosciences. This system, developed with support from the Defense Advanced Research Projects Administration (DARPA) and now owned by Abbott Laboratories, combines broad-range and clade-specific PCR primers to screen for bacteria, viruses, fungi, and protozoa. Simultaneously, it also provides molecular antibiograms by detecting several antibiotic resistance genes, including *mec*, *van*, and *KPC*. In addition, all assays provide relative species abundance information through an internal quantitative control. This microbe ratio is valuable when studying diverse bacterial populations that change over time.

The Ibis technology employs electrospray ionization (ESI) mass spectrometry (MS) before converting amplimers to base compositions. Those base compositions are compared to a library of more than 750,000 entries providing species-level identifications for organisms present at greater than 1–3% of the total microbial bioburden. The detection limits for some high-risk pathogens such as *Staphylococcus aureus* are much lower, meaning they can be identified at 0.01% bioburden or below.

With this system, sample processing does not require enrichment or culture steps, and depends only on basic pipetting skills and an ability to operate instruments. The technology can work with anything from swabs to biopsies. Nucleic acids are isolated directly from specimens. The throughput of these systems is 250 samples per day, meaning one instrument and three technicians could replace the personnel in a typical diagnostic lab (Table 1).

**Some Results Call for Confirmatory Analyses**

Because PCR can yield false-positives, secondary confirmation is recommended, including via 16S fluorescence in situ hybridization (FISH) and deep 16S DNA sequencing. FISH is based on the use of complementary fluorescently labeled DNA probes that bind to ribosomal RNA (rRNA) sequences; these hybridizing pairs of nucleic acids can be visualized using confocal microscopy. The high numbers of ribosomes in bacterial cells ensure exquisite sensitivity. FISH not only detects species but can also help to localize infections.

Advances in parallel sequencing technologies make deep 16S sequencing a useful option for identifying bacteria. This technology uses primers that target 16S rDNA bacterial sequences that are compared to those in databases to determine genus. The barcoding of primers provides for massive multiplexing, wherein hundreds of thousands of 16S amplimers are simultaneously analyzed on instruments such as the Roche 454 Life-sciences Titanium platform. Barcoding reduces costs while boosting throughput. 16S sequencing is not suited for use in clinical diagnostic settings, where individual turnaround times need to be minimized. Like FISH, it serves as an independent tool for confirming results from MS-based diagnostic technologies.

**Evaluating Modern Diagnostic Technologies Firsthand**

Over the past three years, our group at the Center for Genomic Sciences has analyzed more than 4,000 samples from diverse sources to determine whether the PCR-ESI-TOF-MS (Ibis) technology

**TABLE 1. PLEX-ID Workflow**

<table>
<thead>
<tr>
<th>Protocol step</th>
<th>Time required for completion</th>
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</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>30–60 minutes</td>
</tr>
<tr>
<td>Nucleic acid extraction</td>
<td>1–1.5 hours</td>
</tr>
<tr>
<td>PCR setup</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Multiplex amplification</td>
<td>2–3 hours</td>
</tr>
<tr>
<td>PLEX-ID operation</td>
<td>1.5–2 hours</td>
</tr>
<tr>
<td>Total time</td>
<td>5.5–8 hrs</td>
</tr>
</tbody>
</table>

Total time varies depending on the nucleic acid extraction technique used, PCR program differences for different assay plates, and whether automation equipment is utilized.
could replace traditional microbial culture-based diagnostic procedures. As part of this effort, we used 16S FISH for confirmatory testing and deep 16S sequencing as a reference test.

These three DNA-based technologies consistently support one another and outperform culture-based tests. One key finding is that most infections are polymicrobial, which DNA-based tests are better suited to detect.

In general, culture detects about 50% of Staphylococcus aureus infections, 20% of coagulase-negative staphylococcal and streptococcal infections, and other pathogens at even lower rates (Fig. 1). In contrast, all three DNA-based techniques are consistently more sensitive. Further, positive DNA findings correlate with the presence of living organisms because hosts rapidly clear dead bacteria and microbial DNAs below detectable limits.

These technologies might shed light in orthopedics, where the diagnosis and treatment of infections is costly and difficult because so many cases involve bacterial biofilms. Direct and indirect costs for bone and joint health exceed $800 billion per year, with that burden expected to grow. We are using PCR-ESI-TOF-MS to study several types of orthopedic infections, including periprosthetic joint infections (PJIs), traumatic bony nonunions, septic knees, and failed anterior cruciate ligament grafts.

With PCR-ESI-TOF-MS, we identified novel bacteria in many cases of PJIs and then confirmed those findings with FISH and deep 16S sequencing (Fig. 2). In one case, this technology not only detected coagulase-negative staphylococci but also vast numbers of Bacillus cereus, which several European research groups also report can cause orthopedic infections. U.S. diagnostic labs typically consider B. cereus in PJIs a contaminant. However, bacterial FISH and multiple specific PCR and RT-PCR assays confirm B. cereus in this orthopedic case.

With our collaborators at the Rothman Institute of Orthopaedics in Philadelphia, we detected
bacterial pathogens in 85% of supposedly aseptic cases that we reevaluated with PCR-ESI-TOF-MS. Our finding bacteria in these cases underscores the importance of this technology for uncovering previously missed pathogens. We suspect that these cases appear aseptic because the pathogens are slow-growing organisms that do not elicit typical inflammatory responses.

Using this technology to diagnose cases of PJI, septic arthritis of the knee, or other orthopedic conditions could decrease the costs for treating such patients. For example, in 2006 there were more than 1.4 million visits to emergency rooms for symptoms consistent with knee infections, according to the National Hospital Ambulatory Medical Care Survey. In most cases, those patients were placed on antibiotic therapy, which is costly, disrupts the microbiota, and increases the likelihood of selecting for antibiotic-resistant organisms. PCR-ESI-TOF-MS testing technology in hospital clinical diagnostic labs could help to identify uninfected patients who should avoid these treatments.

This diagnostic technology provides other advantages over traditional culture-based diagnostic procedures when dealing with orthopedic trauma and wounded tissues that are vulnerable to opportunistic pathogens. For instance, slow-growing anaerobes can gain access through breached epithelium and then evade the immune system. However, our analysis via PCR-ESI-TOF-MS and FISH of cases involving long bone nonunion revealed different types and percentages of bacteria during different stages of treatment. Additionally, information about bacterial populations in closed versus open fractures is being used by manufacturers to develop implants.

**Diagnosing Chronic Wounds, Avoiding Hospital-Acquired Infections**

PCR-ESI-TOF-MS technology is being used to diagnose nonhealing wounds, the treatment of which is complicated and costly. Antibiotic treatment may eradicate one member of a bacterial community but allow other species to thrive. Because culture-based diagnoses do not provide...
CLUDING those involving patients with cystic fibrosis or recurrent vaginosis, this diagnostic technology can survey the full bacterial and fungal landscape, not merely the culturable members of the microbial community. Continuous monitoring will lead to building patient bacterial profiles that can be targeted with appropriate treatments. Any shift in that profile may indicate a treatment change. Greater knowledge of the microbial ecology provides physicians with a greater capacity to manage their patients effectively. PCR-based techniques can provide information crucial for diagnosing infections and profiling microbial communities throughout treatment.

In managing patients with chronic conditions, preventing hospital-acquired infections (HAIs)
is crucial for their welfare. The cost of treating HAIs, which are transmitted via air, water, and surfaces, accounts for more than $4 billion in health care costs annually. Hospitals could use PCR-ESI-TOF-MS for monitoring nosocomial infections without waiting for regulatory approval. In a 2007 study, environmental surveillance at 20 U.S. hospitals indicated that 70% of water systems and 30% of environmental samples were positive for *Legionella* spp. Increased *Legionella pneumophila* infection rates correlated with contamination of the water supply, illustrating the importance of routinely testing environmental specimens to control HAIs. Such surveillance can pinpoint areas of high bacterial load, enabling hospitals to prevent outbreaks.

Despite the apparent advantages of using second-generation PCR technologies to diagnose infections and monitor microbial communities, no one has conducted a large-scale, double-blind study to compare PCR-ESI-TOF-MS to traditional culture-based diagnostic methods. We urge the scientific clinical community to develop such protocols and to use 16S FISH and deep 16S sequencing for confirmatory purposes. Although European health officials approve this analytic approach, U.S. Food and Drug Agency officials are yet to evaluate any of these bacterial detection assays. Even so, we predict that a decade from now, members of the clinical diagnostic community will wonder why it took so long to replace Petri dishes with PCR.

**Suggested Reading**


