



Forty Years of Molecular Diagnostics for Infectious Diseases

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ABSTRACT Nearly 40 years have elapsed since the invention of the PCR, with its extremely sensitive and specific ability to detect nucleic acids via *in vitro* enzyme-mediated amplification. In turn, more than 2 years have passed since the onset of the coronavirus disease 2019 (COVID-19) pandemic, during which time molecular diagnostics for infectious diseases have assumed a larger global role than ever before. In this context, we review broadly the progression of molecular techniques in clinical microbiology, to their current prominence. Notably, these methods now entail both the detection and quantification of microbial nucleic acids, along with their sequence-based characterization. Overall, we seek to provide a combined perspective on the techniques themselves, as well as how they have come to shape health care at the intersection of technologic innovation, pathophysiologic knowledge, clinical/laboratory logistics, and even financial/regulatory factors.

KEYWORDS PCR, diagnostic molecular microbiology, magnetic beads, real-time quantitative PCR, sequencing, nucleotide array, syndromic panel, point of care, DNA sequencing

MICROBES AND THE DIAGNOSTIC POWER OF DNA

Since the late 19th century, clinical microbiology has evolved alongside our basic understanding of infectious diseases (1). These disciplines trace their roots to the work of Pasteur, Koch, and Lister, from whom Germ Theory introduced the revolutionary concept that particular microbes are responsible for specific infections (2). As we appreciate now more than ever, the relationship between microbes and human physiology can be complex, in ways that are still not fully decoded. Nevertheless, Germ Theory is a foundational principle of modern medicine, and the overarching mission of the clinical microbiology laboratory remains much unchanged: to identify bacterial, fungal, viral, and parasitic pathogens and the diseases they elicit.

For over 100 years, the microscope, microbial culture, and immunodiagnostics served as cumulative sources of laboratory data for diagnosing infections. These techniques remain gold standards for many pathogens, and there is little reason to expect that their role will disappear. What has changed dramatically in recent decades are complementary diagnostic strategies stemming from the advent of molecular biology. In 1944, Avery, MacLeod, and McCarty demonstrated that DNA purified from a type III strain of *Streptococcus pneumoniae* could impart virulence onto a nonpathogenic type II strain (3). The ability of DNA to encode genetic data stems from the structure-function of its component bases, so famously elucidated in 1953 by Watson and Crick (and building on the work of many others) (4). This mechanism for information-storage creates inherent diagnostic possibilities, such as identifying DNA (or RNA) as human versus microbial, predicting microbial phenotypes from their genotypes, and classifying

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organisms taxonomically (5). Of course, to bring these applications to fruition, commensurate tools were needed to detect DNA in a targeted manner.

Accordingly, the last 40 years have witnessed a sea change with the development of ultra-sensitive techniques that leverage DNA/RNA as diagnostic analytes, as molecular assays have evolved from *mere concepts* to *indispensable tools* in the practice of clinical microbiology (6). The advent of genetic (and now genomic/multi-omic) knowledge has created a new normal, redefining the information that laboratories can provide and the speed at which they can provide it. The present article thus serves as an opportunity to synthesize how dramatically molecular testing, now entailing nucleic acid detection, quantification, and sequencing, has come to shape the management of infectious diseases. We seek to provide an integrated review on the techniques themselves, as well as their thematic implications for laboratory operations and patient care. Driven by advances in analytic technology and host-pathogen biology, these themes are critically interconnected with clinical logistics and even financial/regulatory matters, reflecting the pervasive role of molecular diagnostics in general practice and specialized care.

EARLY NONAMPLIFIED PROBE TECHNOLOGY

Probe-based assays, without nucleic acid amplification, were among the first molecular *in vitro* diagnostics (IVDs) approved by the US Food and Drug Administration (FDA) for infectious diseases (7). These technologies generally require “biological amplification” prior to analysis, that is, *in vitro* microbial growth, but without generating amplification products (amplicons) as potential cross-contaminants. Molecular probes constitute single-stranded oligonucleotides, ~15 to 30 bases in length, that hybridize with complementary microbial nucleic acids. They are typically DNA based, although RNA or synthetic DNA mimetics (e.g., peptide nucleic acids) have also been used (8). Probes identify a targeted organism via a sequence that is sufficiently/uniquely conserved among strains of the species, often tracts of rRNA. For detection, they may be conjugated directly or indirectly to a chromogenic enzyme, fluorophore, or radioisotope. Probes can be applied to specimens directly or to fixed/sectioned tissue, including as the basis of *in situ* hybridization by which pathologists can visualize pathogens histologically/cytologically (9).

Within clinical microbiology laboratories, nonamplified probes have traditionally been employed as a substitute for the biochemical identification of microbial isolates or as an adjuvant to culture-based methods. They can be applied to microbial cultures at the first instance of detected growth to yield a more rapid taxonomic identification. Common examples include slowly growing fungi and mycobacteria (10), as well as bacterial/fungal blood cultures (11). Fluorescent *in situ* hybridization (FISH) of positive blood bottles was popularized in the early 2000s, with individual probes directed against common agents of bacteremia. More recently, nonamplified probe technologies for blood cultures have evolved into multiplexed microarrays that identify numerous targets in parallel (12). The utility of molecular probes is not limited to taxonomic identification; they may also characterize antimicrobial resistance, so long as the presence (or absence) of a particular gene/allele is rigorously predictive of a resistance phenotype. Common examples include staphylococcal *mecA*, enterococcal *vanA/vanB*, and various extended-spectrum beta-lactamases and carbapenemases of Gram-negative bacteria (13, 14). These multiplex probe technologies have continued evolving to incorporate other surface- and suspension-based arrays, often in combination with explicit nucleic acid amplification (as discussed further below).

Historically, nucleic acid probes also facilitated the development of *signal-amplification* assays, including hybrid capture and branched chain DNA techniques. Here, the interaction between probe and target is augmented through successive rounds of nucleotide and/or antibody hybridization, maximizing the detectable readout (15). Perhaps the most widespread application of signal amplification for infectious diseases entailed the detection of human papillomavirus in Pap smears (16). In current practice, signal-amplification techniques have largely given way to *target-amplification* of nucleic acid. Nonetheless, advantages

of signal amplification included its ease-of-use and ability to sensitively detect nucleic acid targets without amplicon generation.

THE ADVENT OF NUCLEIC ACID AMPLIFICATION

A clear inflection point in the field of molecular biology, many would argue the true birth of molecular diagnostics, came in the early 1980s, when Mullis developed PCR at the Cetus Corporation (17, 18). PCR is based on the ability of purified DNA polymerase to synthesize double-stranded DNA (dsDNA) *in vitro* from a single-stranded DNA (ssDNA) template and a 5'-oligonucleotide primer. By applying primers to both forward and reverse strands, this activity can selectively amplify a targeted sequence (i.e., the region flanked by the two primers) from extremely complex nucleic acid mixtures. PCR achieves exponential sensitivity, down to single-digit copies of starting template, from the theoretical doubling of amplicon that comes with each temperature cycle. It likewise enjoys remarkable specificity that can accommodate virtually any targeted pathogen, by adapting the primer sequences. The first applications of PCR were technically complicated due to the thermolability of the polymerase then used (*Escherichia coli* DNA polymerase I, Klenow fragment). This created a need to add more Klenow fragment to the reaction after each denaturation step. However, the process was further improved at Cetus by the application of a heat-stable polymerase from the thermophilic bacterium *Thermus aquaticus*, isolated from Yellowstone hot springs, allowing a single reaction mixture to be utilized across all cycles (19). Another key advance was the initial application of reverse transcriptase to PCR specimens (RT-PCR), converting any RNA present to cDNA and allowing PCR to amplify the genomes of RNA viruses or mRNA/rRNA from any microbial target (20).

Various technical advances throughout the 1980/90s likewise helped usher PCR to widespread clinical use (21). The evolution of dedicated laboratory equipment for PCR was crucial, notably the development of the thermocycler. This instrument provides a thermally controlled "heat block" to achieve the desired temperature at each step of the reaction (22). Many modern instruments employ the Peltier effect, which permits heating and cooling of the block by reversing the electric current (23). Thin-walled reaction vessels provide rapid thermal conductivity and equilibration, while heated lids prevent condensation at the top of the vessel (older thermocyclers instead relied on a layer of oil or wax). Second-generation (or "rapid cycle") PCR instruments have incorporated additional features to ensure accelerated temperature ramping (24). These include smaller reaction volumes (down to submicroliter), forced air heating/cooling with microfluidic interfaces, and simplified cycling (e.g., abbreviated steps with combed annealing and extension) (25). These innovations allow for >40 cycle reactions with total processing times less than half an hour.

In broad strokes, diagnostic PCR can be summarized as three component-steps: (i) specimen processing and nucleic acid extraction, (ii) target amplification, and (iii) detection/characterization of amplified product (Fig. 1). Before reviewing detection technologies, we wish to recognize the initial role of specimen extraction. Indeed, the inherent sensitivity of PCR cannot be leveraged without efficiently purifying DNA/RNA from the desired pathogen (in turn present within a clinical specimen). Boom et al. (26) integrated chaotropic specimen lysis with the nucleic acid-binding ability of silica particles, creating an adaptable strategy for extracting, purifying, and concentrating nucleic acids. Manufacturers have developed numerous systems based on this and similar principle, often in combination with magnetic purification and/or filtration, with protocols adapted for diverse microbial targets (27). Instrumentation is now available across varying levels of automation and workload, allowing labs to select the most appropriate methods for their needs.

"VISUALIZING" AND QUANTIFYING AMPLIFICATION PRODUCTS

While PCR can exponentially amplify low-abundance DNA/RNA, its diagnostic utility relies on paired methods for detecting the resultant products (step iii above). Amplicons must be detected by simple, direct, and reproducible means to be leveraged effectively by

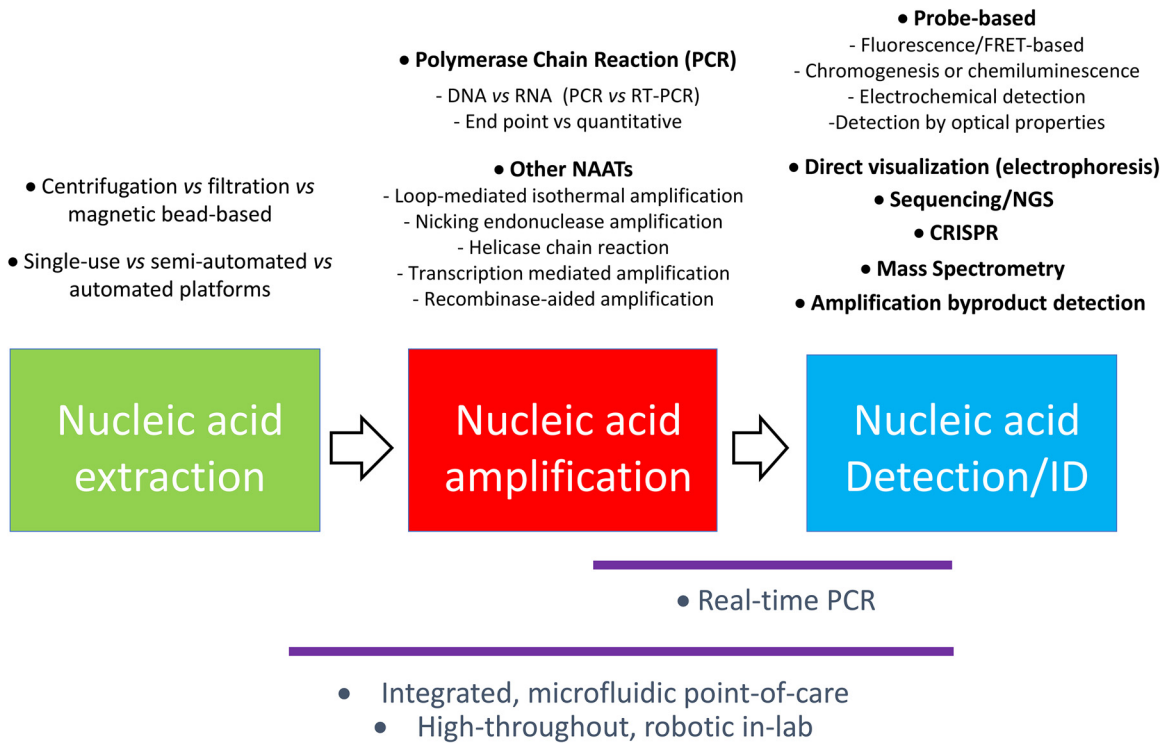


FIG 1 Basic three-step procedure for a nucleic acid amplification assay in clinical microbiology, comprising specimen extraction, RNA/DNA amplification itself, and amplification product detection/identification. For each step (at top), different technical/procedural options employed in clinical settings use are summarized. Important classes of diagnostic technology also combine (conceptually or instrumentation-wise) the component steps together, summarized at bottom. These include qPCR/qRT-PCR, which combine amplification and detection, as well as various high-throughput automated and point-of-care testing platforms, which segue from one step to the next through engineering features.

clinical laboratories. In early diagnostic PCR, the product was electrophoresed through an agarose gel and stained with a DNA-binding fluorescent dye (e.g., ethidium bromide) to visualize amplicons under UV light. This practice allows for direct detection and ensures that amplicons are of the expected size (28). While still widespread for research purposes, this practice is diagnostically suboptimal due to the required effort and open manipulation of amplicons (29). Rather, as an evolution of nonamplified technologies, PCR is combined with molecular probes for combined amplification/detection. The specifics of these composite methods are extremely diverse, but they are connected by their use of one or more labeled oligonucleotides that bind amplicons and facilitate an empirical readout. Many platforms exploit matrix hybridization in both solid and liquid formats (12); methods for signal generation include antibody conjugation and chromogenesis (30), amplicon identification via mass spectrometry (31), and modulation of electronic microcircuits (32). Sequencing has also been employed in amplicon detection, as discussed further below.

Perhaps the most widespread method for PCR detection in clinical microbiology entails fluorescent probes. An early milestone in PCR was the use of fluorescence of to actively monitor amplification. Higuchi et al. (33) pioneered a system that included ethidium bromide within the reaction mixture as a fluorescent agent. The thermocycled reaction was connected fiber optically to a spectrofluorometer; 500-nm light irradiation excited vessels, and the resultant increase in 610-nm emission (from amplicon generation and dye intercalation) was monitored (33). Moving ahead, fluorophore-conjugated oligonucleotides allow for even greater specificity of detection. These techniques generally exploit fluorescent resonance energy transfer (FRET) (34). Here, two fluorophore moieties are only active when in spatial proximity to one another or, alternatively, a fluorophore is inactive when in the presence of a quencher (35). For PCR detection, these phenomena are exploited by conjugating such moieties either to different regions of a single probe or to multiple probes that target adjacent regions of an amplicon.

Hybridization modifies the spatial relationship of the moieties, such that emission only occurs with target amplification.

While there are many variations of this theme, hydrolysis (or “TaqMan”) probes are among the most widespread, exploiting the 5'-exonuclease activity of *T. aquaticus* DNA polymerase (36). During primer extension, this enzyme will cleave any oligonucleotide in its path that is already hybridized to the target. A fluorophore and quencher are conjugated to a probe at opposite ends (5' and 3'), with increasing levels of unquenched fluorescence as amplification progresses. The added value of fluorescent PCR probes for microbial diagnosis lies on multiple fronts. With current instrumentation, the individual steps of amplification, detection, and target confirmation (i.e., via melt-curve analysis) can occur within a single, sealed reaction vessel (37). Not only does this simplify an assay, but it prevents the risk of manipulating amplicons within the laboratory. In addition, the unique emission profiles of different fluorophores (across the visible spectrum) create an ability for multiplexing (38). Distinct amplification processes can be tracked at once, differentiable by the colors of their respective fluorophores. While the extent of such multiplexing is limited by spectral overlap, it can still significantly increase an assay's scope and throughput.

Critically, probe techniques such as TaqMan are conceptually straightforward: with only two primers and one probe per target, this method can be adapted rationally to diverse and/or novel microbial targets. This elegance allows even individual clinical laboratories to validate new laboratory developed tests (LDTs) that meet local needs (39). These “home-brew” reactions are often developed around unique patient populations, uncommon specimen types, or geographically limited pathogens, for which no commercial assays have been promulgated with national regulatory approval (a particular need during the early days of molecular testing). In the United States, for instance, LDTs must be validated according to characteristics dictated by the Clinical Laboratory Improvement Amendments (CLIA), but without FDA approval/clearance. The conduciveness of TaqMan to assay development is particularly relevant to emerging pathogens, as exemplified by the first assays promulgated worldwide for coronavirus disease 2019 (COVID-19) in early 2020 (40).

Beyond these strengths, a critical advantage of fluorescence PCR detection is its ability to detect organisms not just qualitatively, but also quantitatively. For many infections, medical decisions are based not only on the mere presence/absence of a pathogen, but on its abundance. The most common scenarios involve bloodstream viral loads (VL), where physicians utilize viral concentrations to infer the response of a chronic infection to a patient's treatment or immune status (41), as discussed further below. There is likewise an increasing desire for molecular quantification of opportunistic pathogens from inherently nonsterile anatomic sites, such as the respiratory (42) and urogenital tracts (43). Here, the same microbes that elicit frank infections can represent commensal flora or nonspecific findings, and the organism burden may reflect different roles.

For these and other clinical scenarios, fluorescence PCR techniques are particularly well suited. While the endpoint fluorescence of reaction is similar regardless of the initial quantity of target, the time at which fluorescence is detectable is intimately connected to this load. The doubling of amplicon with each cycle creates a log-linear relationship between the initial quantity of target and the cycle number (C_T value) at which fluorescence reaches a detectable threshold. By analyzing calibrators of defined concentrations alongside diagnostic unknowns, pathogen loads may be interpolated from their C_T values and linear regression (41). Alternatively, quantified values can be inferred from a single calibrator, if the amplification efficiency is rigorously validated across a defined range. This process of “real-time” PCR and RT-PCR (qPCR and qRT-PCR, for quantitative) serves as the basis for the majority of quantitative infectious diseases assay, whether commercial platforms with formal regulatory approval or local LDTs. Most recently, digital droplet PCR (ddPCR) has created an alternative approach for the absolute quantification of nucleic acid targets, with the ability for greater accuracy at very low target concentrations. This emulsion technology is based upon massive fractionation of specimen into numerous (up to millions) of

nanoliter-scale droplets, each of which undergoes amplification and microfluidic detection (44), with quantification via Poisson modeling of the fraction of positive droplets.

NUCLEIC ACID AMPLIFICATION OTHER THAN PCR

It is a testament to its groundbreaking nature that the term “PCR” is often used synonymously with “nucleic acid amplification.” We must emphasize, however, that other nucleic acid amplification tests (or NAATs) have been developed that utilize strategies other than PCR, often isothermal methods that do not require temperature cycling. Although a detailed review of these technologies is beyond the scope of this review (45), we note that several methods have been incorporated into popular commercial platforms with broad distribution. These include loop-mediated isothermal amplification (46), nicking endonuclease amplification reaction (47), transcription mediated amplification (48), and the helicase chain reaction (49). Moreover, the COVID-19 pandemic has led to several CRISPR/cas-based assays gaining IVD-status for the first time through *emergency use authorization*, in combination with loop-mediated or recombinase-aided amplification (50). The theoretical upside of isothermal techniques stem from their simplified instrumentation and, in some cases, amplification-times more favorable than rapid-cycle PCR.

COMMON APPLICATIONS OF MICROBIAL NAATS

Forty years into this revolution, the advent of PCR and other NAATs has vastly improved the capabilities of clinical laboratories and shifted the definition of “standard” diagnostic care. NAATs have become an integrated component of guideline-driven practice for common inpatient and outpatient infections. These include upper respiratory tract infections (URIs), such as COVID-19 (51) and influenza (52); acute gastroenteritis (53); sexually transmitted infections (STIs), both symptomatic cases and asymptomatic screening (54); and suspected cases of meningoencephalitis (55). Across these scenarios, molecular methods have largely supplanted traditional culture for viral pathogens, especially given the technical complexity and incubation times of culture (56, 57). With enteric viruses, moreover, most causative agents are not cultivable under standard diagnostic lab conditions. Similar dynamics apply to the utilization of transmission electron microscopy for viral diagnosis, now limited to specialized clinical and public health scenarios (58). NAATs are likewise a standard of care for bacterial whose growth requirements exceed routine capabilities (e.g., *Chlamydia*, *Mycoplasma*, and *Bordetella*.) (59–61). For various parasites, NAATs have increasingly supplanted approaches that rely on microscopic observation (e.g., *Trichomonas*, *Entamoeba*, *Cryptosporidium*), although stool immunoassays also remain in common use (53).

By contrast, the role of molecular diagnostics for more readily cultivated bacteria and fungi is more difficult to characterize in a single, broad stroke. For such species, the relative value of NAATs versus nonmolecular testing depends on the specific organism and anatomic site of infection. In general, though, numerous molecular assays have been developed that seek to provide similar data to routine bacterial/fungal culture, but in a more rapid or straightforward manner. In certain cases, for example, when screening for colonization (e.g., rectal, vaginal, nasal) by opportunistic pathogens or antibiotic resistant strains, NAATs can offer commensurate performance to traditional selective/differential culture and represent a logistically favorable alternative (62). For other infections, the additional sensitivity of culture (down to a single viable organism) dictates that NAATs cannot serve as complete substitutes. However, even here, molecular testing can significantly improve patient care by its ability to serve in a “rule-in” capacity. For instance, a respiratory or cerebrospinal fluid (CSF) specimen that is PCR negative for *Mycobacterium tuberculosis* must still undergo several weeks of culture to evaluate for this organism with total certainty, which is slowly growing and can be present in specimens at extraordinarily low burdens (63). However, a positive result from a rapid PCR allows a clinician to establish a diagnosis of tuberculosis in real time and even adapt therapy to the possibility of drug resistance. The latter is the basis for the Cepheid Xpert MTB/Rif assay, which has helped democratize molecular diagnostics in the developing world (64). Negative results from this same test,

especially when repeatable, can also be leveraged by infection preventionists to indicate a low probability of infectious tuberculosis, prompting discontinuation of airborne precautions (65).

As emphasized above, the need for molecular quantification has likewise become routine across numerous viral pathogens and clinical circumstances. These include chronic infections like human immunodeficiency virus (HIV) (66), hepatitis C virus (HCV) (67), and hepatitis B virus (HBV) (68), for which VL levels can indicate the progression of infection and a patient's response to therapy. These measurements are especially important in light of current antiviral agents, which have made indefinite viral suppression possible for HIV and total cure a reality for HCV. Testing is also indicated for latent viruses within immunocompromised patients, especially stem cell and solid organ transplant recipients, where endogenous reactivation (or even donor-derived infection) can lead to significant morbidity. These include various DNA viruses such as cytomegalovirus (69), Epstein-Barr virus (EBV) (70), BK virus (71), as well as (increasingly recognized for symptomatic patients) adenovirus (72), parvovirus (73), and human herpesviruses 6/7/8 (74). Testing for such viruses in the blood can inform care in several ways. In patients without signs/symptoms, an increasing endogenous viral load can suggest excessive immunosuppression or a nascent pathology not yet recognized clinically (either frank infection or viral-driven neoplasia, in the case of EBV). Conversely, if there is clinical suspicion for disease at a particular anatomic location, a bloodstream VL can help clinicians determine if a given virus is the etiologic agent, especially if that site cannot be directly sampled and tested without an invasive procedure.

For many of these pathogens, commercial qPCR platforms are now available that have undergone FDA trials and formal approval within the United States, often with calibration traceable to the international-unit standards of the World Health Organization (WHO) (75). These processes afford a level of quality assurance and data standardization between individual labs that perform a test. Notoriously, a VL calculated for a specimen can vary significantly between different qPCR assays, unless they are explicitly validated for interassay reproducibility and commutability (76, 77). Such discordance can stem from differences in primer/probe sequences, alternate DNA/RNA extraction methodologies, PCR instrumentation, the source of calibrators that facilitate quantification, and even the fragmentation of circulating nucleic acid for a particular pathogen and specimen type (for example, whole blood versus plasma) (78). At academic institutions, reference labs, and public health facilities, the diversity of NAATs (both qualitative and quantitative) can be far greater, often catering to unusual clinical scenarios. It is again worth emphasizing that these assays are often LDTs, with no extant FDA-approved methods. Naturally, caution is needed when comparing quantitative values among such tests or with externally published data.

SEQUENCING TECHNOLOGY IN MICROBIAL DIAGNOSTICS

While NAATs remain the most common molecular tools within clinical microbiology laboratories, a pathogen's genetic sequence can also provide valuable data. Accordingly, diagnostic sequencing is assuming a growing role in the care of infections. This is especially true given advances in sequencing technology, which has expanded beyond chain-termination methods to include next-generation sequencing (NGS) that simultaneously characterize millions/billions of reads (79). These NGS technologies include both short-read second-generation methods (e.g., Illumina, Ion Torrent), as well as long-read third-generation techniques (e.g., PacBio, Oxford Nanopore) (80). Across platforms, most sequence-based diagnostics now in clinical use have been developed by individual laboratories as LDTs. Nevertheless, several sequencing assays have achieved national IVD status for the evaluation of HIV (81), including the first NGS platforms (82).

Sequencing can be employed to characterize microbial DNA/RNA purified directly from clinical specimens, as well as from individual strains already isolated in culture. For instance, while bacteria/fungi isolates are identified mainly by surrogate methods (e.g., biochemical phenotypes and mass spectrometry), the basis of taxonomy lies in DNA (5). When surrogate methods are insufficient, sequencing of microbial housekeeping genes

can provide an even higher standard. These include the 16S rRNA gene of bacteria and the ribosomal internal transcribed sequence (ITS) of fungi, although other loci may be necessary for taxonomic resolution, depending on the organism (83). Strain-level sequencing of various loci can also play a valuable role in tracing outbreaks, by its ability to gauge relatedness/clonality among strains (84). Given the advent of NGS, assays based on whole-genome sequencing (WGS) are playing increasing role in defining outbreaks (85) and characterizing pathogen epidemiology (86), especially by public health labs.

Moreover, sequencing can evaluate an organism's relevant clinical phenotypes on a strain-to-strain level, including to guide antimicrobial therapy. It is particularly valuable for pathogens with challenging growth properties (slow, cumbersome, or posing a bio-safety risk). HIV is a well-established example with its protease, reverse transcriptase, and integrase loci, for which polymorphisms correlate with antiretroviral resistance (66, 87). In general, viruses represent attractive targets for antimicrobial genotyping due to their small genomes (88, 89). For bacteria and fungi, WGS likewise raises possibilities for *in silico* antibiograms of cultured isolates or uncultured specimens (90). For example, with *M. tuberculosis* and potentially other mycobacterial species, polymorphisms in defined genes correlate strongly with phenotypic resistance to common agents (91, 92). Ongoing work seeks to expand this paradigm to other organisms, including when resistance is not comprehensively mapped or is a complex function of interacting loci. Of note, multilocus and machine-learning models are increasingly applied to WGS data for antimicrobial susceptibility predictions, with hopes of establishing correlations for diagnostic purposes (93).

Molecular assays are not necessarily divided between *amplification/detection* and *sequence-based characterization*. In a growing trend, sequencing may be exploited to detect the presence of a pathogen within a clinical specimen, while simultaneously characterizing its identity. Across its variations, this strategy has been described as *broad-range* testing, *agnostic* testing, and diagnostic *metagenomics* (94). For instance, American LDTs and European IVDs have been established around pan-bacterial (95), pan-mycobacterial (96), and pan-fungal PCR (97). Here, the aforementioned loci (16S, ITS, etc.) are targeted in PCR-reactions against total nucleic acid extracted from tissue (fresh or fixed) or fluid specimens. Amplicons are sequenced to confirm detection and identify of the offending pathogen(s). While this approach is not necessarily more sensitive or specific than traditional microbial culture or target-specific NAATs (at least across all organisms/sites), it can provide valuable supplemental data in certain circumstances, for instance if clinical/microscopic findings suggest infection but corresponding cultures yield no growth.

Going further, nucleic acids extracted from clinical specimens can be subjected directly to NGS-based deep sequencing (98, 99). Although most reads will be host derived, if only a small fraction corresponds to a pathogen, their presence can suggest an etiologic role. Plasma represents a prominent specimen-type for these metagenomic analyses, although detection of microbial nucleic acid in the blood is not the same as bacteremia (or other "emias") (100). Instead, microbial reads often reflect cell-free nucleic acid originating from an infected focus within the body, accessing the circulation via the extracellular space. Thus, low levels of microbial DNA/RNAemia may be present without viable or intact circulating organisms. Commensurate techniques have been launched for CSF, bronchoalveolar lavage (BAL) fluid (101), and synovial specimens (102). Granted, the development, quality assurance, and reporting of these assays may be substantially more complex than for other molecular diagnostics, especially with regard to reference standards, controls, microbial quantification, and the need for validated sequence databases. Discerning commensal or transient flora (or even background signal) from opportunistic pathogens can also pose a challenge. The clinical interpretation of metagenomic data requires care, as challenges can arise both from the novelty of techniques and the personalized nature of the data. At present, such testing is only offered by a limited number of reference centers, although clinical successes are well documented (98, 99). Future metagenomic testing may be further

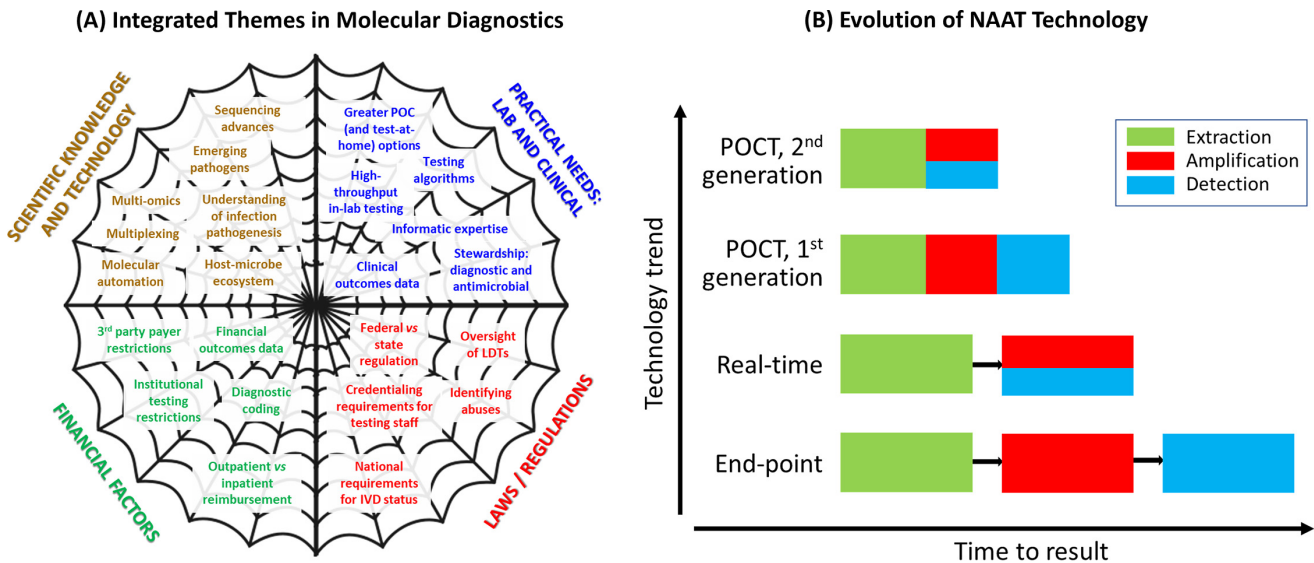


FIG 2 (A) Integrated themes in molecular diagnostics. A web of interconnected factors now guide the application of molecular diagnostics to infectious disease care. With examples provided here, these include advances in the diagnostic technology itself and our understanding of the pathobiology of infections and host-microbe interactions; practical and logistical considerations in both the laboratory and clinic; prominent matters of health care finance and reimbursement; and the regulatory/legal framework surrounding diagnostic testing around the world. (B) Evolution of NAAT Technology. Republished with permission from reference 159. POCT, point-of-care test.

augmented through its theorized ability to predict antimicrobial susceptibility (103) or even the host response via transcriptomics (104).

PROMINENT THEMES IN MOLECULAR MICROBIAL DIAGNOSTICS

Across so many methodologies and pathogens, molecular infectious disease testing has become incredibly diverse. Nevertheless, this landscape is notable for a number of overarching themes, ones that demonstrate great interconnectedness between technologic advances, our understanding of microbial pathogenesis, and key meta-phenomena that guide real-world diagnostic applications (Fig. 2A). First and foremost is a desire for operational simplicity: although their engineering is increasingly complex, molecular diagnostic devices are strongly trending toward greater ease-of-use. Naturally, the role of molecular testing as routine care carries higher expectations around throughput and turnaround time (105). In short, a modern microbiology laboratory would not be viable if all molecular work were conducted manually by subspecialized technologists.

As a result, commercial molecular platforms are increasingly designed with an emphasis on automation and sample-to-result capabilities, analogous to total laboratory automation in culture-based diagnostic microbiology. Here, robotic liquid handling can integrate nucleic acid extraction with amplification, detection, and even reporting, minimizing the needs for direct specimen manipulation (106). The goal is that even laboratories without dedicated teams of molecular microbiologists can support a large menu of molecular tests (although experts are still needed to identify and troubleshoot problems that do arise, preventing testing from becoming a complete “black box”). Accordingly, a common practice for major manufacturers is the development of automated platforms, to which a portfolio of individual NAATs can be applied, each with their own IVD status (107–109). In fact, the popularity of such instruments can alter the layout of clinical laboratories. Traditionally, molecular laboratories have placed tremendous emphasis on their spatial workflows. A clear separation was maintained between preamplification and postamplification areas, often with one-way traffic to prevent molecular cross-contamination. Although preventing molecular contamination is still a

key component of a lab's quality plan, the use of self-contained automation significantly reduces potential risks (29).

The need for simplicity in molecular diagnostics extends further, beyond the laboratory itself. One of the most noteworthy trends is the desire to implement molecular testing at the point-of-care (POC), including in clinics and urgent care settings (110). POC testing in microbiology is not a new concept, as rapid antigen tests have existed for years for upper respiratory viruses (influenza and respiratory syncytial virus) and streptococcal pharyngitis. In general, POC diagnostics can expedite definitive therapy, prevent unnecessary therapy (i.e., antimicrobial stewardship), and facilitate infection prevention practices. Molecular POC tests offer further advantages over their traditional immunoassay counterparts, especially with their improved sensitivity and negative predictive value (111). For instance, to rule out infection definitively, a negative influenza or streptococcal antigen result must be followed by an additional in-lab testing, molecular or culture based. By contrast, a negative molecular POC test for these agents can be self-sufficient as a rule-out, eliminating the need for empirical treatment while follow-up results are pending.

Whereas in-lab molecular platforms are increasingly designed for throughput, POC devices are engineered for rapid, *ad hoc* use at any given time. They incorporate nucleic acid extraction, amplification, and detection together into an integrated and sealed cartridge, making it simple, rapid, and safe (Fig. 2B). Operational simplicity is of even greater importance for POC instruments, including to achieve regulatory approval ("CLIA-waived" status within the United States). Low complexity is vital, as the clinicians who serve as end-users often have no formal training in molecular techniques or diagnostic quality monitoring. Even before COVID-19 (112), several molecular POC tests for respiratory pathogens were launched in recent years (111), as well as the first such platforms for STIs (113), and the scope of molecular POC testing is only expected to grow. As predicted in 2004 by Raoult et al. (114), microbial diagnostics may become increasingly polarized: random-access assays conducted directly in clinics (or even with an at-home component) with batched, automated panels in central laboratories.

These same POC considerations are likewise vital in the context of global health and diagnostic availability for the developing world. In 2003, the WHO proposed the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered) as goals for diagnostics in resource-limited settings, with a particular emphasis on tropical (e.g., malaria, tuberculosis) and sexually transmitted (HIV, syphilis) infections (115). While a modicum of instrumentation is generally required for molecular testing, these principles can still inform the development of molecular diagnostics for the entire global community (116). Important practical considerations were articulated recently by the proposed RE-ASSURED criteria, including flexible powering and connectivity to mobile devices for reporting (117).

Of course, any discussion of molecular infectious disease testing would be incomplete without addressing the emergence of highly multiplexed "syndromic" assays (118). For various infections, the differential diagnosis can include numerous pathogens that cannot be differentiated clinically and, instead, require parallel testing for each organism. Obtaining this information rapidly can facilitate both targeted escalation and de-escalation of care, facilitating antimicrobial stewardship (119). The number of candidate pathogens often exceeds the multiplexing limits of for traditional multicolor qPCR. Instead, dedicated methods are required that segregate potential amplicons spatially or by some other labeling. Several PCR-driven platforms are now in common clinical use; these include instruments whose probe-based detection relies on: spatially arrayed fluorophores (120); surface arrays with optical detection of metallic nanoparticles (121); disruption of impedance across microcircuits (32); and modulation of magnetic resonance relaxation properties (122). Importantly, such instruments must incorporate dedicated microfluidics that link nucleic acid extraction, amplification, and detection within (ideally) a self-contained device.

Syndromic assays are utilized broadly in clinical scenarios that include respiratory infections, both URIs, with testing performed on nasal/nasopharyngeal swabs (123),

and pneumonia, performed on BAL fluid (124); acute diarrheal illness, performed on stool (125); meningoen­cephalitis, performed on cerebrospinal fluid (126); and blood-stream infections. Depending on the platform, the latter may entail analysis of either blood itself (127) or the initial microbial growth within blood-culture bottles (128). Additional syndromic assays are under active development and evaluation, including for joint (129) and urinary tract infections (130). For one platform (FilmArray), a respiratory panel has been granted CLIA-waived status for POC utilization (131).

While multiplex panels carry great analytic power, their implementation has not come without concomitant challenges. A frequent scenario, particularly for respiratory and gastrointestinal panels, involves the detection of multiple targets and whether this reflects active coinfection (132). In fact, a key caveat of *all molecular testing* is that detection (in itself) cannot differentiate the nucleic acid of viable pathogens from the “molecular remnants” of resolved/treated infections. Similar challenges pertain to distinguishing infection from asymptomatic colonization. This potential ambiguity is inherent when interpreting any molecular data, but it is particularly relevant when many targets are detectable at once and in patients with high pretest probability (e.g., children and URIs). Similarly, assays developed as initial tests-of-diagnosis are not always appropriate as molecular tests-of-cure, a dynamic that attained prominence for COVID-19 and the desire to use negative follow-up results to inform contact restrictions (133). Paradoxically, the high analytic sensitivity of such tests can reduce their ability to discern resolved infection.

Even when the clinical interpretation of a multiplex assay is not in doubt, additional questions can arise surrounding issues of clinical actionability, proper utilization, and diagnostic stewardship (134). To offer a simplified example, *does an immunocompetent adult require multiplex stool testing for a limited episode of nonbloody diarrhea?* Most clinicians would answer *no*, as a definitive diagnosis would not alter the course of supportive care. In this light, various society-level recommendations now include suggested parameters for syndromic testing, based on a patient’s clinical presentation and risk factors (135, 136). Individual institutions have likewise promulgated algorithms for when syndromic testing is permitted, for instance, in combination with a patient’s age, testing rationale, or other lab data (137). For better or worse, these questions are also intrinsically linked to health care economics. Although financial models differ significantly across locations worldwide, syndromic molecular testing has not escaped the attention of payers when deciding which tests are reimbursable, generating a great deal of discussion among major stakeholders (138). In the United States, in fact, molecular CPT (current procedural terminology) codes for syndromic infections are now stratified by the number of multiplexed pathogens.

These questions of stewardship and the reimbursement highlight an even broader issue surrounding molecular microbial diagnostics: an increased recognition of the need for *outcomes* research (139). Traditionally, research and development efforts for molecular assays have focuses on their analytic/diagnostic performance; in other words, characteristics that are validated *before* a test is implemented clinically. Once a test is launched, though, questions often remain around its ideal utilization and the ultimate consequences for patient care and health care costs, inside and outside the lab (101). More diagnostic data are not necessarily “good data” if they do not improve outcomes or conserve resources in an evidence-based manner, although defining the attributable outcomes of a test is not always straightforward. Nonetheless, recent years have witnessed a growing body of outcomes-based research in clinical microbiology, with syndromic panels as one of the most popular topics (140, 141). Similar questions apply to metagenomic and other emerging NGS assays, with a need to systematically define their role alongside nonmolecular and targeted molecular assays (142). A prominent viewpoint, also in light of emerging data, is that these workflows might complement traditional methods, but certainly not yet replace them (143).

Returning to the analytic dimension of testing, one must recognize that molecular analytes for infectious diseases could extend beyond nucleic acids (144). In *sensu lato*,

molecular diagnostics encompass not only genomic, but also metabolomic (145), transcriptomic (146), and proteomic technology (147). In fact, the now ubiquitous application of proteomics to bacterial/fungal identification, via matrix-assisted laser desorption/ionization-time of flight mass spectrometry, is a testament to the power of -omic technology (148). Such advances have raised hopes that diagnostics might one day incorporate multipartite molecular signatures of host and pathogen, although they also underscore a need for greater fluency in bioinformatics, medical informatics, and other computational techniques in clinical microbiology. This reality is already experienced by labs that have implemented NGS pipelines with their massive quantities of data (149).

These new paradigms could even transcend traditional definitions of microbial disease. The last decade has witnessed an explosion in our understanding on the role of the microbiome in human pathophysiology (150). Vast associations have now been identified between the population structure of the microbiome (within the gut and elsewhere) and clinical conditions throughout the body (151, 152). This *pathobiome* view of health and disease is founded on complex host-microbe relationships at the physical, metabolic, and inflammatory level, ones that often fall outside Koch's historic criteria for microbial disease. However, characterizing a patient's microbiome could be valuable diagnostically if it creates an ability to impact management, in essence, the human microbial ecosystem as a biomarker (153, 154). In this vein, the utility of such testing will depend not only the associations themselves (with traditional metrics like sensitivity, specificity, and receiver-operator characteristics) but on the aforementioned concept of actionability (155, 156). In essence, *will defining a patient's microbiome allow clinicians to augment care through personalized medicine?* Although his concept of diagnostic microbiome testing remains in its infancy, it raises the exciting idea that clinical microbiology may ultimately evolve beyond just infectious diseases.

Finally, for both established and nascent technologies, one must consider the impact of evolving legal/regulatory requirements. For any location, the current *status quo* of diagnostic oversight is never immutable. Within the United States, for instance, assays must be characterized according to CLIA-defined performance categories, either broadly through FDA premarket review or as single-site LDTs. Future legislation could alter this dynamic, however, including the proposed VALID Act and its potential expansion of national LDT oversight (157). In the European Community, moreover, the diagnostic sector is preparing for implementation of the *In Vitro* Diagnostics Medical Devices Regulation 2017/746, which expands premarket requirements for assays (158). While such changes are not limited to the care of infectious diseases, this area could be among the most profoundly affected given the now pervasive role of molecular testing.

CONCLUDING PERSPECTIVES

While the fundamental mission of clinical microbiology laboratories remains steady, the scope of their work has metamorphized over the past 40 years and molecular diagnostics are at the root of this change. These assays have transformed the care of infectious diseases, from routine conditions encountered in daily practice to rare scenarios managed by specialists. Ongoing advances will only continue to shape this landscape, not only from a technical perspective, but with interconnected logistical, financial, and regulatory factors that are critical to modern health care delivery. In short, this molecular diagnostic revolution shows no signs of slowing.

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