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# Diagnostic Molecular Microbiology: A 2013 Snapshot

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## KEYWORDS

- Molecular microbiology • PCR • Probe tests
- Rapid molecular diagnosis of infections • Multiplex PCR panels • MALDI TOF
- Nuclear magnetic resonance

## KEY POINTS

- In 2013, diagnostic molecular testing has a large and increasing role in the diagnosis of infectious diseases.
- It has evolved significantly since the first probe tests were FDA approved in the early 1990s.
- It has evolved beyond PCR or even RT-PCR to include highly multiplexed PCR carried out in microfluidic pouch systems, matrix-assisted laser desorption/ionization time of flight, and nuclear magnetic resonance.

## INTRODUCTION

When Kary Mullis developed the polymerase chain reaction (PCR) in 1983, its potential benefits were obvious to clinical microbiologists: faster, cheaper, more accurate detection and enumeration of all organisms in a specimen, without waiting for culture. We also wanted simultaneous antimicrobial susceptibility testing. Our dreams are now coming true. Multiplex arrays are approved or in development for the diagnosis of respiratory and gastrointestinal infections direct from patient specimens within less than an hour. An array was FDA cleared in August, 2013, that can detect common bacterial and fungal agents of bloodstream infections, as well as several important antibiotic-resistant genes, within about an hour after the culture bottle turns positive. Microbiology lines are available, starting with automated plate streakers and ending with molecular identification of organisms grown on solid media. Humans must still view the culture plates, perhaps on a television screen, and select colonies to analyze.

“Cheaper” is an ambiguous target. Microbiology laboratories are diagnostic facilities that drive subsequent therapy. Increased laboratory costs for more rapid

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Clin Lab Med 33 (2013) 787–803  
<http://dx.doi.org/10.1016/j.cll.2013.08.003>

[labmed.theclinics.com](http://labmed.theclinics.com)

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microbial identification have been shown to result in earlier use of appropriate antibiotics, shorter lengths of hospital stay, and better outcomes, decreasing overall health care costs.<sup>1-3</sup> Diagnosis of persistent human papilloma virus infections followed by appropriate therapeutic interventions should reduce the incidence of cervical carcinomas, the cost of treatment, and the attributable morbidity and mortality.

New technologies have enabled microbiologic investigations that were not included in our original dreams. NextGen sequencing can detect and quantify populations of organisms in patient specimens. This raises the possibility of distinguishing pathogenic organisms, present in high numbers, from colonizers that are generally presumed to be present in lower numbers. Certain colonic organism profiles appear to correlate with the development of cardiovascular disease.<sup>4</sup> A patient's colonic flora could be analyzed, and if the profile were unfavorable, the bacteria could be eradicated and replaced.

Tests in use in 2013 have evolved significantly and will continue to do so. Thus, this article is a snapshot of rapidly changing diagnostic microbiology laboratory techniques. Because of space constraints, emphasis has been placed on tests with high market share in diagnostic microbiology and on those with technologies that are personally regarded by the authors as particularly interesting. The role of specimen processing in concentrating nucleic acid targets and removing inhibitors of amplification is largely neglected, despite its important role in the sensitivity of the assay. Most techniques mentioned here involve RT-PCR, unless otherwise specified. RT-PCR lowers the incidence of amplicon contamination in the laboratory, and has allowed many nucleic acid amplification techniques to come "out of the closet," but does not prevent specimen contamination. The authors have also attempted to select only one current citation to support most points, and these selections are arbitrary. Failure to mention a publication, technique, or trade name should not be construed as denigrating that article, technique, or manufacturer.

## PROBE TECHNIQUES

The first molecular diagnostic tests approved by the Food and Drug Administration (FDA) were probe techniques. The probes were synthesized by molecular techniques, but the clinical laboratory performed only hybridization and detection. Many probe tests are still in wide use today because they fill important niches. Some involve novel detection methodologies.

### *Hybridization-Protection Assays*

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Among the first FDA-approved molecular tests were the Gen-Probe ([San Diego, CA], which became a wholly owned subsidiary of Hologic [Bedford, MA] in 2012). Pace 2 probe hybridization protection techniques for the diagnosis of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) from patient specimens. They have been largely replaced by more sensitive amplification tests. A number of their AccuProbe culture confirmation tests remain available. Among them are *Mycobacterium tuberculosis* (TB) complex, *Mycobacterium avium*, *Mycobacterium intracellulare* (separately or together), *Mycobacterium kansasii*, and *Mycobacterium goodii*. In addition, there are 3 tests for dimorphic fungi: *Histoplasma capsulatum* (which also detects *H capsulatum* var. *dubosi*), *Blastomyces dermatitidis* (which also detects *Paracoccidioides brasiliensis*), and *Coccidioides immitis*. These and other tests available from the same manufacturer all use most of the same reagents and instrumentation, which facilitates the use of multiple assays in the same laboratory.

These tests succeed because they target ribosomal RNA (rRNA), which is present in up to  $10^5$  copies per organism. In bacterial ribosomes, there are common sequences, as well as genus-specific and species-specific sequences. The culture confirmation tests remain viable because they are intended to detect organisms in visible colonies or in liquid medium with detectable growth. Thus, 2 amplification steps have already been performed by nature. Sensitivities reported in the Hologic/Gen-Probe package inserts range from 98% to 100%.

At development, these assays were novel: they were nonradioactive and performed totally in solution, with 1 sample transfer step and no nucleic acid extraction. The hybridization-protection assays are based on the differential sensitivity of the acridinium ester, which is used to distinguish the relatively labile ester on nonhybridized probes from the more stable form in DNA-RNA hybrids. This detection system is also used in this manufacturer's nucleic acid amplification tests (NAATs). All the AccuProbe assays are similar but the nucleic acid release steps are variable, depending on the ease of disruption of the organism.

Many laboratories have used the mycobacterial probes for approximately 20 years, and have found no reason to argue with the FDA-approved sensitivity and specificity, both of which exceed 99.6%.<sup>5</sup> Although *Mycobacterium celatum* in high concentrations also reacts with the TB complex probe,<sup>6</sup> we have seen only 1 in 20 years (Fairfax, unpublished data, 2013).

The mycobacterial probe tests are particularly valuable when used in conjunction with liquid medium or 7H11 thin-plate techniques<sup>7</sup> used for the rapid detection and identification of mycobacteria required by the College of American Pathologists (Northfield, IL). Thin-plate colonies can be probed the day they are detected, and their morphology can be used as a guide to selection of the appropriate probe. This represents significant time and money saving, although it does not eliminate the need to grow *M tuberculosis* for susceptibility testing. If there is only one visible colony and if the probe result is negative, whether due to inadequate sample, improper probe selection, or growth of an organism not recognized by the probe, one must wait for the colony to regrow before further analysis (Fairfax and Salimnia, unpublished data). Cultures in Middlebrook 7H9 broth must be AFB stained to confirm the presence of mycobacteria<sup>8</sup> and then probed with all 4 probes.

### **Hybrid Capture Technique**

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Since the 1940s, Pap smears have contributed to great advances in the prevention and diagnosis of cervical carcinoma. In the 1980s and 1990s, it was recognized that infection with human papilloma virus (HPV) is necessary but not sufficient for the development of cervical carcinoma. More than 100 HPV genotypes exist, of which 13 are associated with a high risk of cervical carcinoma (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). More than 70% of sexually active women become infected with HPV. Most infections, even with high-risk organisms, resolve without apparent sequelae. Why others progress is still unknown.<sup>9</sup> The Digene Hybrid Capture 2 (hc2; Qiagen, Gaithersburg, MD) is FDA approved for primary screening, and to determine whether women with atypical squamous cells of undetermined significance should be subjected to colposcopy. It may be used with the Cytoc PreservCyt Solution for the Cytoc ThinPrep Pap Test (Hologic).<sup>10</sup>

In the first step, the patient specimen is allowed to react with a pool of RNA probes designed to hybridize specifically to the DNA of high-risk HPV strains. Antibody to RNA/DNA hybrids coats the wells of a microtiter plate and captures any hybrids. After washing away unbound specimen and reagents, detector antihybrid antibodies, each conjugated with multiple molecules of alkaline phosphatase, bind to each captured

target, amplifying the signal. A colorless substrate for the alkaline phosphatase is added and chemiluminescence develops proportional to the amount of second antibody bound.

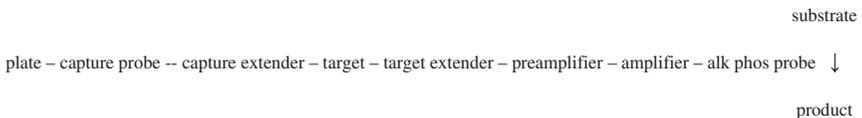
More sensitive tests, typically involving target amplification, have been devised, but most clinical outcomes data are available with hc2. The need for more sensitivity has been questioned, as hc2 has already been shown to be sensitive, but not highly specific, for the development of cervical intraepithelial neoplasia or overt malignancy. The presence of high-risk DNA below the detection limit of hc2 appears to be associated with a low risk for malignancy.<sup>9</sup> However, new information suggests that infections acquired in the early years of sexual activity may reactivate with aging.<sup>11</sup> If this is supported by further studies, more sensitive assays might be indicated. There is also a suggestion that unusual HPV strains may cause cervical carcinoma or precursor lesions in restricted populations.<sup>12</sup> Thus, the strains included in the assay may need periodic review.

### ***bDNA***

Several generations of the Versant branched-chain (b) DNA signal amplification viral load tests (Bayer Corp, Berkeley, CA) have been used for determination of cytomegalovirus (CMV), HIV, and hepatitis B virus (HBV) viral loads for 15 to 20 years. The most recent bDNA HIV viral load assay is version 3.0, and the current lower limit of quantification (LLQ) is approximately 8 to 10 nucleic acid targets per reaction, or about 73 copies/mL,<sup>13</sup> in contrast to the LLQ of 10 copies/mL for the latest nucleic acid amplification viral load techniques (see later in this article). Although overnight incubation has been standard, a new algorithm that can be performed in 9 hours has been published.<sup>14</sup>

The bDNA technique resembles a “molecular tree” (Fig. 1). The wells of microtiter plates are coated with capture probes, which bind the target extender, then the target. Three more hybridization steps occur, with significant amplification at each. In the last, multiple alkaline phosphatase-coupled probes bind to the branched amplifier probes. Each enzyme converts multiple molecules of its colorless substrate into colored end product, allowing the detection of small numbers of initial targets.

bDNA is unaffected by amplicon contamination (because there are no amplicons) and relatively insensitive to inhibitors that plague many NAATs. It is also less sensitive to problems resulting from freezing specimens in plasma preparation (PP) tubes.<sup>15</sup> This mode of specimen transport is no longer recommended for amplification-based viral load determinations, as DNA escaping from the gel matrix in the PP tube can increase the apparent viral load by hundreds of copies/mL, which was unnoticeable when the viral loads were routinely in the thousands or tens of thousands,<sup>15</sup> but which is unacceptable today, when viral loads of <20 are frequently reported (see later in this article).



**Fig. 1.** The bDNA “molecular tree” technique.

### ***Peptide Nucleic Acid Fluorescence In Situ Hybridization***

Peptide nucleic acid fluorescence in situ hybridization (PNA-FISH) (AdvanDx, Inc, Woburn, MA) has accelerated the diagnosis of sepsis. Common agents of sepsis

can be identified in about an hour after the blood culture bottle has been Gram stained. Although behaving like a standard FISH assay, the PNA-FISH probes consist of an uncharged peptide backbone to which the bases are attached. This is thought to allow the probes to enter the permeabilized bacterial cell more easily and then bind more tightly to the negatively charged rRNA target. Numerous publications confirm that use of this technique for rapid identification of the common organisms growing in the blood culture bottles improves antibiotic stewardship and shortens length of stay.<sup>1,2</sup> Depending on the patient, identification of a coagulase-negative *Staphylococcus* may facilitate discontinuation of antibiotics and early discharge (Salimnia and Fairfax, unpublished results, 2013).

Our high-throughput laboratory (>200 blood cultures per day) was an early adapter of the staphylococcal, enterococcal, and candida probes, although we do not currently use those for gram-negative organisms because we determined that the test's ability to identify only 2 or 3 organisms was not cost effective for our complex patient population. We run the gram positive and fungal assays once per shift. In general, the identifications are congruent with culture and identification on the MicroScan (WalkAway 96si; Siemens HealthCare Diagnostics, West Sacramento, CA). Rare misses have occurred in mixed infections due to failure to detect very low numbers of *Staphylococcus aureus* against the background of a much higher number of coagulase-negative *Staphylococcus*, or similar low numbers of *Enterococcus faecalis* in a background of other *Enterococcus* spp. On review of the slides, the previously undetected organism was seen (Salimnia, unpublished results). This no longer occurs as our technologists are alert to this possibility. In a few, rare situations, the PNA-FISH and the MicroScan have disagreed. These discordant results have been resolved in favor of PNA-FISH by 16S rRNA sequencing.<sup>16</sup> Depending on how one resolves the results of the mixed cultures described previously, the sensitivity and specificity of PNA-FISH in our laboratory approximate 100%.

Red/green fluorescent probe kits at this time include coagulase-negative *Staphylococcus* (green) versus *S aureus* (red), *Enterococcus* sp (red) versus *E faecalis* (green), *Escherichia coli* (green) versus *Pseudomonas aeruginosa* (red), and *Candida albicans* (green) versus *Candida glabrata* (red). Two tests use 3-color (red, yellow, green; traffic light) fluorescence: gram-negative rods (*E coli* [green], *Klebsiella pneumoniae* [yellow], and *P aeruginosa* [red]) and *Candida*. In the *Candida* assay, *C albicans* and *Candida parapsilosis*, both susceptible to most antifungals, fluoresce green, *Candida tropicalis* fluoresces yellow, and both *C glabrata* and *Candida krusei* fluoresce red. Also approved are 20-minute, 2-color tests for *Staphylococcus* sp versus *S aureus* and *Enterococcus* sp versus *E faecalis*, potentially allowing the reporting of a preliminary identification at the same time as the Gram stain. A universal bacterial probe is available as an analyte-specific reagent (ASR), as are specific probes for *Acinetobacter*, *Candida dublinensis*, and *C parapsilosis* (AdvanDx Web Products section of site, queried September 5, 2013).

### **Affirm VPIII Microbial Identification Test**

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The VPIII probe test (Becton Dickinson [BD], Franklin Lakes, NJ) is intended for the diagnosis of vaginitis/vaginosis, conditions causing millions of physician visits annually. It detects rRNA from *Gardnerella vaginalis*, used as an indicator for bacterial vaginosis (BV), *C albicans*, and *Trichomonas vaginalis*. The sensitivity has been adjusted to avoid giving positive results with low concentrations of *G vaginalis* and *C albicans*, which often colonize the normal vagina. It is formatted to be performed in a cassette superficially resembling the lateral flow tests used for the serologic detection of influenza or rotavirus antigens and was the first FDA-approved molecular test based on

lateral flow. After collection on a proprietary swab, the specimen is lysed to release the nucleic acids, buffered to stabilize the nucleic acids and establish stringency, and added to the cassette, which is incubated at the proper temperature for nucleic acid hybridization. The cassette contains 5 “beads,” each coupled to a capture probe: 1 for each of the 3 analytes, plus positive and negative controls. Next, enzyme-linked detector probes bind to specific sequences on the target organism’s rRNA. Unbound sample components and probes are washed away. A colorless substrate is converted to a blue product if sufficient target and detector probe have bound. A blue “bead” indicates a positive result.<sup>17</sup>

Numerous publications have revealed that health care providers are significantly less accurate than VPIII for the diagnosis of significant candidiasis, and *T vaginalis*. The role of the VPIII in the diagnosis of BV is still the subject of some debate, as *G vaginalis* is found both in the presence and the absence of BV. In an analysis of results from our laboratory, mixed infections were common. Of those patients positive for *T vaginalis*, almost 2/3 were positive for one or both of the other organisms.<sup>18</sup> FDA-approved NAATs with higher sensitivity and a higher price are also available for *T vaginalis* (Hologic/Gen-Probe).<sup>19</sup>

## QUALITATIVE NAATS

This section concentrates on tests that give a positive or negative result for 1, 2, or 3 analytes. Highly multiplexed NAATs are discussed in a section entitled “Highly Multiplexed PCR Panels.”

### ***Sexually Transmitted Disease Testing***

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Culture, immunoassay, and probe tests for both NG and CT are less sensitive than NAATs, and NAATs have been the standard of care since the late 1990s, although certain jurisdictions require culture for legal cases. Despite the relatively long history of NAAT use, the testing modalities are still undergoing significant changes that are mentioned later in this article. It should be noted that, because of updates in targets and instrumentation, previously published comparisons may no longer apply.

Although NAAT is generally assumed to mean PCR, this is not always the case: when PCR was patent protected, other innovative amplification techniques were developed. Two of these are isothermal, avoiding the requirement for expensive thermal cyclers: strand-displacement amplification (SDA; BD Probe Tec ET and Q<sup>x</sup>) and transcription-mediated amplification (TMA; Aptima Combo 2; Hologic Gen-Probe). These are both described in detail in the methods article. The TMA assay targets organism-specific rRNA sequences in each organism: 23S rRNA for CT and 16S rRNA for NG. It begins with target capture on magnetic beads, and ends with the hybridization protection assay similar to those previously described for the AccuProbe tests, although each species of detector probe is attached to a different acridinium ester. One flashes very rapidly and its chemiluminescence attenuates before the other begins to fluoresce (glow). Thus, they can be detected sequentially in the same tube.<sup>20</sup>

The BD SDA CT assay targets an open reading frame in the cryptic CT plasmid.<sup>21</sup> The testing is very complicated to describe (see the methods article), but much simpler to perform, especially on automated instrumentation. The manufacturers of both isothermal amplification tests sell high-throughput analyzers using the same (or modified, as in SDA Q<sup>x</sup>) technology. Two PCR-based tests, by Roche (Roche Cobas Amplicor [RCA]; Roche Molecular Diagnostics, Pleasanton, CA) and Abbott (Abbott RealTime [ART]; Abbott Molecular, Des Plaines, IL) CT/NG assay (on their m2000 automated platform) are also major players in the huge market for diagnosis

of CT and NG. Both PCR-based CT tests originally targeted sequences in the cryptic plasmid.<sup>22</sup>

The older tests are FDA approved for cervical swabs, genital swabs, and urine. Several are approved for physician-collected or self-collected vaginal swabs. Both TMA and SDA are approved for use with the specimen collected for the Thin Prep PAP smear technique,<sup>20,23</sup> although the newer versions may not yet be approved for all specimen types. To prevent contamination, the sample for nucleic acid amplification must be removed before processing the cytology sample for cellular analysis.<sup>20,23</sup> The specificities of all the assays are comparable, although the RCA has been reported to detect nongonorrhoeae *Neisseria*,<sup>24,25</sup> and their sensitivities appear to be adequate for genital specimens, with TMA appearing slightly more sensitive than standard SDA, both of which may be slightly better than either PCR assay.<sup>21,22,24,26</sup> In general, the sensitivities of all 4 tests are lower for CT than for NG, perhaps due to a lower organism burden for CT.<sup>22</sup>

The high-throughput, automated BD SDA assays for NG and CT (ProbeTec Q<sup>x</sup>) performed in extracted mode on their Viper System have improved sensitivity. This may be due partly to target changes and in part to contaminants being removed by use of a magnetic, ferric oxide bead-capture system. The ferric oxide technique could be generalizable because it does not require the presence of specific target sequences. Briefly, the bacteria are lysed in a high-pH solution. The pH is lowered to generate a positive charge on the ferric oxide particles, which bind the negatively charged nucleic acids. The particles are magnetically captured and washed, and the bound DNA is eluted by raising the pH. The extracted DNA is buffered to a pH appropriate for the amplification assay.<sup>21,23</sup>

Recently, several studies have been performed to extend these tests to rectal and oropharyngeal specimens. Although not yet FDA approved for these sources, their sensitivities and specificities appear adequate with the exception of oropharyngeal NG by RCA.<sup>24,25</sup> The RCA test has been shown to detect nongonorrhoeal *Neisseriaceae* and is not appropriate for nongenital testing.<sup>24,25</sup> Perhaps in response to this, Roche has introduced a new version of its STD tests (Cobas 4800), which targets the DR-9 region of NG, improving its specificity and also has changes, described later in this article, that improve its ability to test for CT.<sup>24</sup> This is not yet approved in the United States.

How long we will be able to continue using these tests is unclear, because problems have arisen with both CT and NG. A CT strain with a 377-base pair (bp) deletion in the cryptic plasmid that includes the original targets of both PCR tests has been detected in Sweden, where in 2009 it comprised 24% to 45% of CT-positive specimens.<sup>27,28</sup> The organism is rare in the rest of Europe and has not been detected in the United States.<sup>28</sup> Roche and Abbott have both modified their assays to include a second target sequence: *omp1* for Roche and a second region of the cryptic plasmid for Abbott.<sup>24</sup>

NG resistance to each recommended drug regimen has occurred after wide use of that antibiotic; resistance to cefepime been detected and is becoming more prevalent in the United States.<sup>29</sup> Transportation kits for these molecular tests are not compatible with culture and susceptibility testing, which is also significantly less sensitive. Thus, it appears that further molecular analysis of specimens that test positive for NG may be required to detect antibiotic-resistant genes. Such tests are not commercially available. The sequences best included in such assays remain to be determined and probably will need to be changed with time.

Additional tests for other urogenital pathogens are being added to the testing formats. TMA testing for *T vaginalis* was recently FDA approved and is more sensitive

than VP11.<sup>19</sup> Testing for HSV 1 and 2 from swabs of external anogenital lesions is also available from BD in their Q<sup>x</sup> format.<sup>30</sup>

### **Other Qualitative Assays**

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Hologic-Gen-Probe manufactures several widely used qualitative, TMA-based microbiology tests; included among these are a direct test for *M tuberculosis* and the Procleix Ultrio Plus assay (Ultrio), which has also been licensed to Novartis (Emoryville, CA). This latter assay (without the Plus) has long been approved for detection of HIV and HCV in specimens from blood donors and from organ donors, both living and deceased, but is not intended for the diagnostic workup of the diseases in the general population. Testing a pool of samples from up to 16 blood donor units is approved for blood bank testing. Recently, the FDA approved the inclusion of HBV in this assay, and it has been renamed Ultrio Plus. It contains several HIV targets that allow it to detect HIV-1 (several strains) and HIV-2. Because test results are generally negative and high sensitivity is necessary, inclusion of an internal control is essential. This uses the “flash technology,” while a positive result gives rise to a “glow” (see previously). Components of positive pools are retested individually and then by assays for each individual analyte.<sup>31</sup>

A recent article contains a listing of more than 135 published RT-PCR assays developed for detection of 32 species of bacteria.<sup>32</sup> This list does not include multiplexed tests or assays for viruses. The investigator acknowledged that the list is incomplete and commented that most are laboratory-developed tests, with only 35 (counted from his list) being commercially available, whether FDA approved or not. This emphasizes the need for more rapid commercial development and FDA-approval methods.

Although cyber green, a nonspecific detector, is still used occasionally, 3 main molecular detection systems are used: dual hybridization (fluorescent resonance energy transfer [FRET]), TaqMan, and molecular beacon. The FRET detector consists of 2 different probes, complementary to adjacent sequences on the target amplicon, and each attached to a different fluor. When activated by incident UV light, the first fluor emits energy at an unmonitored wavelength. If the second probe is bound adjacent to it, the energy is transferred to the second fluor, which then emits light at the wavelength monitored by the sensor. At the end of the PCR assay, a melting curve for the amplicon-probe complex can be generated. The melting temperature ( $T_m$ ) is characteristic of each amplicon-probe combination. If there is a mismatch between the probe and the amplicon, the melting temperature decreases.<sup>33</sup> The  $T_m$  difference has been exploited in a test that distinguishes between herpes simplex 1 and 2 (Roche). The PCR primers bind sequences common to both viruses within the HSV DNA polymerase gene. The amplicon detector probes match the HSV-2 sequence, which differs from that of HSV-1 by 2 bp. Melting curve analysis reveals a reproducible  $T_m$  for HSV-2 that is about 10°C higher than that exhibited by HSV-1.<sup>34</sup> Occasional mutant HSV strains have been detected that have intermediate melting temperatures. These can be reported as positive for HSV, but the type is not clear. Types 1 and 2 could be distinguished by sequencing the amplicons, by using an assay employing a different target sequence, or by the  $T_m$  of the amplicon, which is said to be 0.9°C higher for the HSV-2 variant than the type 1 variant.<sup>35</sup>

The MultiCode RTx system uses an unusual PCR amplification technique in which no detector probe is used and in which the fluorescence actually decreases as amplification progresses. It was developed by EraGen BioSciences, Inc (Madison, WI; acquired by Luminex Corporation [Austin, TX] in 2011). Their Multicode-RTx Herpes simplex virus 1 and 2 kit was FDA approved in 2011. MultiCode RTx assays use 2 unusual nucleotide bases iC (2-deoxy-5-methylisocytidine) and iG (2'-deoxy-isoguanoside) that base

pair only to one another and that are efficiently incorporated into PCR products. iG is put at the 5' end of the RTx primers with a fluor covalently linked nearby. The reaction mix contains iC covalently linked to a quencher. In the initial cycle amplification, the iG, with its attached fluor, appears at the 5' end of the nascent amplicon strands. When these serve as templates for copying in the other direction, an iC, with its attached quencher is added to the 3' end of the new strand, opposite the iG and its fluor. The approximation of fluor and quencher decreases the fluorescence. In melting curve analysis, fluorescence increases as the amplicons melt, and the  $T_m$  allows determination of the nature of the analyte.<sup>36</sup> Although these RT-PCR assays are qualitative, detection is based on the determination of a CT, as with any other standard RT-PCR. Comparison of CTs can be used to provide a rough quantification of the amount of target DNA.<sup>37</sup>

## QUANTITATIVE TECHNIQUES

Because of space constraints, comments on quantitative PCR in clinical microbiology are confined mainly to NAATs used for HIV viral load testing, although there are also FDA-approved quantitative RT-PCR assays for HCV, HBV, and recently for CMV. The trend is to make the assays referable to a World Health Organization international standard. Quantitative techniques have advanced significantly since the early days when Alice Huang first demonstrated that quantitative PCR was possible (for a detailed discussion and review of the literature, see Fairfax and Salimnia<sup>33</sup>).

RT-PCR is inherently semiquantitative. Theoretically, one can construct a standard curve of copy number versus CT and determine the quantity of analyte in a patient specimen by referring to the curve. However, variations in extraction efficiency and the presence of inhibitors can introduce significant errors, particularly at low levels of analyte, when one is attempting to distinguish "only a few" from "none." One relatively straightforward method to overcome this problem includes the addition of a control target or quantitation standard (QS) into the patient sample before extraction. The target and the QS are extracted, amplified, and detected together, controlling for the extraction and for any inhibitors present. The ideal QS should be the same size and base composition as the target to be quantified, with the same primer binding sites. It should differ enough in sequence that the detector probe or probes for the target do not bind to it, and its own detector probe should fluoresce at a different wavelength from that of the target. Then, quantitation should be a simple mathematical calculation.<sup>33</sup> However, at low target concentrations, the standard curve is no longer straight. Roche has incorporated complex mathematical calculations into its recent viral load assays to account for this divergence.

The statistics of small numbers impact the detection of fewer than 10 targets per reaction mix. What is called "digital PCR" exploits this. For each specimen, multiple reactions are conducted in such small volumes (emulsified droplets, for example) that some contain no targets, whereas others will contain only one or a few. From the volume of sample in each reaction droplet, the number of positive and negative reactions, and the Poisson distribution, one can calculate the number of targets in the original sample. No FDA-approved assays are yet based on this intriguing technique.

Conversations have recently begun about possible "cures" of HIV infection, and as highly active antiretroviral therapy and improved plasma HIV viral load techniques converge, discussions have ensued about how to evaluate residual virus in well-controlled or possibly cured individuals. The most sensitive assays for determination of low-level infection are likely to be assays for HIV DNA copies in single cells. How to determine which ones are replication-competent is crucial to this discussion. Aside

from circulating CD4 cells, what cells should be investigated is unclear at this time. This active area of investigation was recently reviewed by Strain and Richman,<sup>38</sup> who discussed very low level contamination, signal-to-noise ratios in PCR testing, single-cell PCR, and other techniques that are beyond the scope of this article.

With respect to HIV quantification in plasma, further considerations affect testing and result interpretation. The most obvious problem results from the increase in sensitivity as the assays improve. Patients who were told that their virus levels were “undetectable,” suddenly have quantifiable virus. Time-consuming correlations between viral load and prognosis have to be redone for each new, more sensitive assay. Sequence differences between the numerous organism strains and the inherent mutability of the organisms also make accurate quantification difficult, especially for RNA viruses, such as HIV and HCV. One must target a stable sequence. However, the viral RNA polymerase enzymes are error prone and lack proofreading activity. Changes in the genetic sequence, particularly in the primer or probe-binding sites, may reduce detected viral load. Minority quasi-species also cause problems (see later in this article). It appears that more than one target will be necessary for future assays.

Because approval and release of diagnostic tests by different manufacturers are not coordinated, it is difficult to find articles comparing the performance of the latest offerings by different companies. Two current assays for HIV quantification in the United States are the Roche Cobas AmpliPrep Cobas TaqMan HIV viral load version 2.0 (CAP/CTM2) and the Abbott RealTime HIV-1 assay (ART HIV). The 2 assays have different lower limits of quantitation (LLQs), 20 c/mL for CAP/CTM2 and 40 c/mL for ART HIV, which introduces difficulties in comparison. They have, however, been compared in 2 recent articles.<sup>39,40</sup> Sire and colleagues<sup>39</sup> extrapolated the ART HIV curves, and found that 10 of 17 specimens that were quantifiable by the CAP/CTM2 but not by the ART HIV could actually be quantified. The assays correlated well ( $r = 0.96$ <sup>38</sup>), although CAP/CTM2 was more than 0.5 log<sub>10</sub> higher than the ART HIV in 20% of 51 samples, whereas the ART HIV was more than 0.5 log<sub>10</sub> higher in only 2 of them.<sup>39</sup> Whose result is more accurate remains to be determined.

## HIGHLY MULTIPLEXED PCR PANELS

This section focuses only on highly multiplexed assays, roughly defined as those detecting more than 10 targets. Although many assays for individual etiology agents of disease are available, testing for individual organisms is often uninformative. Numerous viral and some bacterial agents of respiratory disease cause similar symptoms. It is usually not obvious which agent or agents are infecting a given patient. Even at the peak of an influenza epidemic, an individual may be infected with respiratory syncytial virus instead or concurrently. Furthermore, mixed infections are more common than many had imagined.<sup>41,42</sup> Some virus infections are treatable, and others have different isolation requirements if the patient is hospitalized.<sup>42</sup> Thus, molecular panels that could detect multiple etiologic agents of diseases clearly are desirable. Four multiplex respiratory panels are FDA approved at this writing: Luminex xTAG RVP (xRVP) and RVP Fast (xRVFP) (Luminex Diagnostics, Toronto, Canada); Film Array Respiratory Panel (FARP; BioFire Diagnostics [formerly Idaho Technologies], Salt Lake City, UT), and eSensor Respiratory Panel (eSRP) (GenMark Diagnostics, Carlsbad, CA). The FARP has the most targets, including 3 bacteria. One head-to-head comparison of all 4 test modalities was recently published.<sup>43</sup> The various analytes differ in sensitivity and specificity, but this study found that the eSRP had 100% sensitivity for all analytes compared. The xRVP was second, and more sensitive than the xRVFP, which was similar to the FARP. These results will require confirmation

by other researchers, as Babady and colleagues<sup>41</sup> found the FARP to be more sensitive than the xRVPF for many analytes. All tests are capable of detecting mixed infections.

Ultimately the final decision as to which test to implement in one's laboratory may come down to questions of cost, hands-on time, complexity, and convenience. The FARP provides a result within about 1 hour, requiring only minutes of hands-on time, but can handle only 1 sample at a time. It seems ideally suited to a medium-sized laboratory where technologists without specialized molecular expertise can perform the tests. Some larger laboratories have bought multiple instruments to facilitate throughput. But surge capacity may be lacking. The others are batch instruments, but they require sample extraction before testing, and amplicon manipulation afterward, which may confine them to a "PCR laboratory." Each handles 21 samples per run. None is suitable for 2 runs per shift, although staggered technologist start times could allow it with the xRVPF. The xRVP and eSRP require 7 to 8 hours for results.<sup>43</sup>

A problem with panels is that they tend to maximize the number of analytes detected in their initial offering, based on instrument constraints. Adding something new seems to require that something old be removed. FDA reapproval requires significant financial outlays for any change, and new respiratory viruses keep appearing. Newly detected viruses since 2001 include human metapneumoviruses (2001), severe acute respiratory syndrome (SARS; 2003), human bocavirus (2005), new influenza A viruses (H1N1, 2009; H7N9, 2013), and now, Middle East respiratory syndrome corona virus (MERS-CoV; 2012), the latter 2 of which apparently have not (yet) appeared in the United States but apparently have a high mortality rate.<sup>44–46</sup> The human metapneumoviruses and human bocavirus are included in some of the previously mentioned assays. It is not clear whether MERS-CoV can be detected by the coronavirus detector systems in these assays, but if so, it could not be distinguished from those that cause more common respiratory infections. Thus, keeping panels updated may prove difficult.

New multiplex panels are in development for viral, parasitic, and bacterial agents of gastrointestinal diseases, and CNS infections. A BioFire assay for usual agents of bloodstream infections was FDA approved in August 2013. At this time, it appears that the gastrointestinal panels will be direct-from-the-specimen assays, whereas the blood culture panels should be run from positive culture bottles (which may take up to 5 days to become positive). Some can detect several antibiotic resistance genes as well as infecting bacteria.

## NEXT-GENERATION SEQUENCING

The advances in sequencing technology known as NextGen sequencing (NGS) have led to significant advances in basic sciences and clinical laboratory medicine, including microbiology. Currently available NGS techniques are based on using clonal amplicons for parallel multistrand sequencing. The combination of high-speed and high-throughput data analysis has made NGS an excellent tool to take clinical analysis of nucleic acid sequences to a new level. With NGS, it is possible to detect and quantify quasispecies of HIV, HCV, or HBV circulating at low levels in the blood of infected patients and to learn more about their roles in the development of resistance and associated treatment failures. This was difficult or impossible by using methods mentioned previously. Despite increasing numbers of articles on the applications of NGS, this system has not found its way into routine clinical microbiology testing because of lack of FDA approval, high cost, and availability of alternatives.<sup>47</sup>

NGS has significantly improved our ability to study the composition of entire communities of microorganisms (metagenomics) without organism culture. NGS simultaneously provides the sequences of genetic material from members of the entire microbial community. Analysis of these data allows determination of the relative proportions of organisms within the biome analyzed. It can also determine gene expression and metabolic pathway utilization in a microbial community. Human-microbial metagenomics studies are focused on understanding the relationship between commensal microbial communities in health and disease,<sup>48</sup> including cardiovascular disease.<sup>4</sup> NGS has also been applied to rapid investigations of outbreaks in hospital settings.<sup>49</sup>

### MASS SPECTROSCOPY IN MICROBIOLOGY

Although “molecular diagnostics” is generally assumed to imply nucleic acid–based methods, mass spectroscopy (MS) has been used in microbiology since the 1970s. At that time, MS was used almost exclusively for the identification of anaerobes by analysis of volatile or volatilized short-chain organic acids. New MS techniques provide a general tool for the identification of microorganisms growing in colonies on culture plates. This requires 2 to 5 minutes and has the potential to improve significantly the turnaround time for microbiology culture reports and to reduce labor costs, especially when coupled with other laboratory automation that is now becoming available. Up-front costs are high, but rapidity of results can impact antibiotic usage and patient outcomes, shortening hospital stays and lowering total costs.<sup>3</sup>

The new technology is matrix-assisted laser desorption/ionization time of flight (MALDI TOF). Currently, there are 2 MALDI TOF instruments available in the United States for rapid identification of microorganisms. One, developed by Shimadzu Scientific Instruments (Columbia, MD) and licensed to BioMerieux (Durham, NC), has just been FDA-approved. The other can be obtained directly from its manufacturer (Bruker Daltonics, Bellerica, MA), who has also licensed it to both Siemens Healthcare Diagnostics (Tarrytown, NY) and to BD. However, the great benefit of easy, rapid, and accurate identification of microorganisms has encouraged some laboratories to purchase these systems and perform their own validations before FDA clearance.

Microorganism identification by MALDI TOF is based on the fact that each microorganism has its unique protein signature (PS). Bacterial cells are fixed to a matrix and exposed to a laser beam that releases and ionizes the proteins. These enter a vacuum column and move toward the detector based on charge and mass. The PS is checked for a match against a rapidly accumulating database derived from different genera and species of microorganisms. Different instruments have different databases.

MALDI TOF can identify microorganisms, including aerobic/anaerobic bacteria, mycobacteria, and fungi. The analysis of organisms with tougher cell walls, such as gram-positive bacteria, yeasts, and fungi, apparently requires a slight modification of procedure.<sup>50</sup> Hundreds of abstracts and articles attest to its ability to rapidly and accurately identify bacteria and fungi to the genus and species level.

MALDI TOF for the identification of etiologic agents of sepsis generally cannot be performed until the pathogen has first been grown in blood culture bottles and subcultured onto standard solid media. These steps are time consuming and lead to the use of broad-spectrum, empiric antibiotics. To increase speed of identification, protocols have been developed to use MALDI TOF directly from newly positive blood culture bottles.<sup>51</sup> Data are also accumulating illustrating the ability of MALDI TOF to identify some antibiotic-resistant organisms.<sup>52</sup> However, more work is needed in this area

before MALDI TOF could be considered as a valid alternative to routine antibiotic susceptibility methods.

Other systems that also use MS have been tested for their ability for rapid microorganism identification. Two examples are briefly mentioned: laser-induced breakdown spectroscopy (LIBS) and Raman spectroscopy (RS). LIBS can identify microorganisms in a very short time based on determination of their elemental compositions. The system uses a strong laser beam pulse that atomizes the content of bacterial cells. Light emitted from these “high-temperature sparks” is collected and dispersed. The atoms present in the specimen are identified by peaks in the atomic emission spectrum. The ratios of the intensities of these peaks form a “spectral fingerprint” that is unique to the bacterial genus and species (LIBS spectrum). This is compared with a database to allow identification of bacterial genus/species.<sup>53</sup> RS can also rapidly identify bacteria to the strain level. It uses a visible or near-infrared laser that provides a reproducible molecular spectrum from the whole bacterial cell, which is unique for each bacterial species.<sup>54</sup>

### LATE BREAKER TECHNIQUES

While this article was in preparation, 2 articles using a novel technique were published.<sup>55,56</sup> Chung and colleagues<sup>55</sup> used polystyrene beads coated with capture probe sequences common to all 16S bacterial rRNA sequences. Asymmetric PCR on the specimen was performed using 16S bacterial rRNA as a target. The PCR product was captured by the capture probes and probed with organism-specific detector probes complementary to species-specific regions of the rRNA molecules. The detector probes are covalently linked to magnetic nanoparticles. The bound magnetic nanoparticles are detected by faster relaxation of their signal in a micronuclear magnetic resonance system. The assay is carried out in a microfluidic chip, requires 2 hours and can be carried out with only 2  $\mu$ L of sample. It can reportedly detect 1 bacterial cell, as well as multiple infections, and can be applied directly to blood and aspirated body fluids. It has been adapted to detect the mRNAs of *mecA* (which codes for PBP2a, the enzyme responsible for methicillin-resistance in *S aureus*), and the Pantone-Valentine leukocidin, which is found in the most common of the community-associated methicillin-resistant *S aureus*. The instrumentation could be used for point of care testing.

The article by Liong and colleagues<sup>56</sup> focuses on the diagnosis of *M tuberculosis*. This assay is similar to the one by Chung and colleagues<sup>55</sup> and is also performed in a microfluidic chip. Sputum specimens are liquefied and loaded into the chip where all steps of the assay are performed, and the nuclear magnetic resonance (NMR) signal relaxation is measured at the end. The assay is also capable of detecting mutations associated with drug resistance.

If future research confirms and extends these findings, all phases of our wildest 1983 dreams may actually be coming true in 2013.

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