Rapid Detection and Identification of Bacterial Pathogens Using Novel Molecular Technologies: Infection Control and Beyond

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The rapid detection and reporting of antimicrobial-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and multidrug-resistant *Mycobacterium tuberculosis*, is a challenge for the clinical microbiology laboratory. Molecular-based diagnostic tests can provide data on the presence of methicillin-resistant *S. aureus* in the nares in \( \approx 1 \) h, whereas testing for the *vanA* and *vanB* resistance genes in enterococci isolated from perirectal samples can be completed in \( \approx 4 \) h. Novel pyrosequencing assays can provide data regarding the presence of multidrug-resistant *M. tuberculosis* directly from positive mycobacterial broth cultures in \(<1\) day. These data can assist physicians in both therapeutic and infection control decisions.

In the movie *Top Gun*, Tom Cruise’s character, Pete Mitchell (otherwise known as “Maverick”), remarks “I feel the need … the need for speed.” This is a sentiment felt by many infectious diseases clinicians and clinical microbiologists, particularly when confronted with a patient who is seriously ill, getting progressively worse, and for whom an infectious etiology has yet to be identified. The need for speed also is a factor when (1) a physician awaits the results of standardized antimicrobial susceptibility testing for an infectious agent that has been isolated from a patient whose condition is deteriorating clinically (despite receipt of empirical therapy) and (2) an infection control practitioner awaits the results of tests to determine whether a patient is colonized or infected with methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant enterococci (VRE) and requires isolation. It is in these types of situations that molecular diagnostic methods can provide the data needed more rapidly and, in many cases, more cost effectively than traditional culture methods.

In recent years, there has been a growing emphasis on the use of molecular methods to detect not just infectious agents but also antimicrobial-resistance genes carried by microorganisms [1]. These studies usually focus on 1 of 4 applications: (1) detecting antimicrobial-resistance genes in organisms directly in clinical samples (e.g., detecting the *vanA* vancomycin-resistance gene in *Enterococcus faecium* present in material from perirectal swabs); (2) detecting antimicrobial-resistance genes or mutations associated with antimicrobial resistance in fastidious organisms (e.g., *Mycobacterium tuberculosis*) prior to the availability of standardized susceptibility test results, which may require several weeks; (3) arbitrating borderline antimicrobial-resistant test results that could significantly impact therapy (e.g., detecting *mecA* in borderline oxacillin-resistant *S. aureus* isolates); and (4) predicting antimicrobial resistance when no standardized in vitro testing method is available (e.g., for confirming the susceptibility of *Helicobacter pylori* to clarithromycin) [1]. To speed the detection of antimicrobial-resistant microorganisms in particular, several new technologies have been introduced in clinical microbiology laboratories during the past few years. In this article, I will explore molecular technologies that can be particularly useful for infection control decisions, including the detection of MRSA, VRE, and multidrug-resistant *M. tuberculosis* (MDR-TB).
MOLECULAR DIAGNOSTICS AND THE U.S. FOOD AND DRUG ADMINISTRATION (FDA)

Molecular diagnostic assays used in clinical microbiology laboratories can be divided into 3 major categories: FDA-cleared assays; analyte-specific reagents (ASRs), which are not formally cleared by the FDA; and in-house research protocols (often involving PCR assays and frequently referred to as “home brew assays”). Stated more simply, tests can be divided into (1) commercially prepared, prepackaged kits with instructions on how to perform the test (FDA-cleared assays); (2) kits with no instructions, but usually with data available in the medical literature or on another institution’s Web site regarding the accuracy and precision of the test (ASRs); and (3) assays that are developed and validated in house. FDA-cleared assays typically have undergone rigorous review and often have performance data from multiple investigation sites published in the peer-reviewed literature. However, laboratories still need to validate the accuracy of FDA-cleared kits in-house before the test can be used to report results of patients’ samples. ASRs (e.g., PCR primers and detection probes) are typically manufactured by a diagnostic company using “good manufacturing practices” but are distributed to users without protocols for test performance and are not packaged in kits that contain all of the necessary buffers and controls. The clinical utility of an ASR does not have to be validated in prospective multisite trials before marketing. Protocols for the use of ASRs are available in the literature or on Web sites. In-house validation assays require more-extensive data than are required for FDA-cleared assays. Home brew assays for infectious agents are widely used and often provide invaluable data, particularly for more unusual infectious agents [2]. However, extensive in-house validation is needed before such assays can be used for clinical specimens. Thus, home brew assays reported in the literature by one laboratory may be difficult for other laboratories to implement because of a lack of the available specimens and expertise needed to perform the appropriate validation studies. The FDA requires laboratories to add a disclaimer to reports involving tests that use ASRs and home brew assays. The mandatory language for this disclaimer is as follows: “This test was developed and its performance characteristics determined by the (laboratory name). It has not been cleared or approved by the U.S. FDA [3].”

PEPTIDE NUCLEIC ACID FLUORESCENT IN SITU HYBRIDIZATION (PNA-FISH)

The simplest of the new molecular technologies are the PNA-FISH probes for identification of organisms directly in positive blood cultures [4]. Peptide nucleic acids have the advantage of rapid hybridization kinetics and, when coupled with fluorescent reporter molecules, offer a sensitive way to identify S. aureus [5], Enterococcus faecalis [6], or Candida albicans [7] in positive blood culture samples, typically within a few hours after a positive blood culture result is obtained. Additional probes for gram-negative organisms, including Escherichia coli and Pseudomonas aeruginosa, also have been reported [4]. Forrest et al. [8] reported a study in which the impact of PNA-FISH probes for rapidly differentiating S. aureus from coagulase-negative staphylococci (CoNS) in blood culture samples containing clusters of gram-positive cocci was evaluated. These investigators studied 223 episodes of bacteraemia that resulted in 223 positive blood cultures. One hundred and thirty-nine of the positive blood culture samples were tested with PNA-FISH probes to differentiate those that contained S. aureus from those that contained CoNS. The goal was to identify those blood culture samples containing CoNS that were likely to be contaminants and, therefore, did not require initiation of vancomycin therapy. An antimicrobial team evaluated the PNA-FISH data daily and made recommendations regarding vancomycin therapy to the attending physicians. A matched control sample of 84 positive blood culture samples containing clusters of gram-positive cocci was not tested by PNA-FISH but was processed using the standard microbiologic methods and usual reporting protocols (i.e., no enhanced notification of results). There was no statistical difference between the case patients and control subjects with regard to age, sex, hospital location, or diagnosis based on codes from the International Classification of Diseases, 9th edition. The PNA-FISH tests were performed once per day and required ~3 h to complete. Although patients were stratified by intensive care unit (ICU) location and non-ICU location, the former group was small, and no statistically significant differences were noted between the PNA-FISH and control groups. However, use of PNA-FISH in conjunction with the input of the antimicrobial team resulted in a significant reduction in the median length of stay of non-ICU patients, compared with a control group of patients for whom the PNA-FISH test was not used (P<.05). A trend towards decreased vancomycin use and earlier discharge was also noted in the non-ICU patients in the PNA-FISH group; however, the data did not reach statistical significance. The cost savings in the non-ICU group was ~$4000 per patient, which was attributed primarily to shorter durations of stay. The antimicrobial team was able to use the negative PNA-FISH results (which indicated that the blood culture sample contained CoNS) to either prevent or limit vancomycin therapy in patients for whom the positive culture results likely represented contamination. (Patients with non-removable intravascular devices, for whom CoNS may represent true bacteraemia, were excluded from the study). Two additional studies that showed the positive impact of using PNA-FISH probes focused on optimizing vancomycin use in a hospital by...
differentiating positive blood culture samples containing *E. faecalis* from those containing *E. faecium* [6] and on cost savings in antifungal drugs, realized by rapid identification of *C. albicans* in blood cultures [9]. The PNA-FISH probes have additional value when coupled with ASRs for rapid detection of *meca* (in blood cultures positive for *S. aureus*) or of the combination of the *vanA* and *vanB* vancomycin-resistance genes (when *E. faecalis* is identified in a blood culture).

**REAL-TIME PCR ASSAYS**

An emerging method for the detection and identification of a variety of infectious agents in clinical laboratories is real-time PCR [2]. This modification of the traditional PCR assay was developed to improve the sensitivity, specificity, and speed of detecting PCR amplification products. Traditional PCR assays often used agarose gels or immunoassays to detect amplification products. This added 1–3 h to the amplification procedure and increased the risk of contaminating the laboratory with amplification products that could lead to false-positive results in future assays. In response, real-time assays were developed in which the accumulation of amplification products is measured as they develop by fluorescence resonance emission transfer probes, molecular beacons, or TaqMan probes (Roche Molecular Systems) in a closed system that reduces contamination [2]. A molecular beacon is a single-stranded probe with a recognition sequence in the interior of a self-annealing molecule that contains a reporter molecular on one end and a quencher on the other (figure 1). If the probe comes in contact with a PCR product with a complementary nucleic acid sequence, the probe opens up and binds to the PCR product, thus separating the fluorescence-reported dye from the quencher, which results in a measurable fluorescent signal. The accumulating fluorescent signal is measured by the instrument as the amplification reaction occurs (i.e., in real time), thereby obviating the need for opening the reaction tubes and running agarose gels for amplification product detection. Similarly, dual fluorescence resonance emission transfer probes (one with a reporter and the other with a quencher) and TaqMan probes can be used in real-time PCR assays for detection of products.

One example of a real-time PCR assay that is available as an FDA-cleared commercial product and is particularly useful for infection control is the rapid detection of MRSA directly in nasal swab samples [10]. This assay, which couples PCR primers that are specific for *meca* and the *S. aureus*–specific gene orfX, uses a series of detection probes to account for the variants of the staphylococcal cassette chromosome mec elements [11]. The test was evaluated by Warren et al. [12] who screened nares swabs from 288 patients for MRSA using the rapid assay, direct culture, and culture following enrichment in broth. Fifty-nine samples had positive results by all 3 methods, 6 had positive results only by direct culture, and 7 had positive results by culture only after enrichment. The sensitivity, specificity, and positive and negative predictive values of the real-time PCR assay were 91.7%, 93.5%, 82.5%, and 97.1%, respectively. In this study, the assay required 1.5 h to process 8–10 samples. Wren et al. [13] also evaluated a commercial amplification test for direct detection of MRSA in 1211 nasal swabs and compared the amplification results to the results of culture on mannitol salt agar plates containing 4 μg/mL of oxacillin. Samples showing discrepant results were retested after placing the original swab in enrichment broth overnight prior to subculture on solid media. The sensitivity and specificity of the amplification assay were 95.0% and 98.8% respectively, with positive and negative predictive values of 84.4% and 99.6%, respectively. No CoNS were falsely identified by the PCR assay, although 4.6% of the positive PCR samples contained methicillin-susceptible *S. aureus*. Results were available in <3 h. A smaller study of 331 nasal specimens collected from 162 patients, conducted by Huletsky et al. [11] and using the same commercial assay as was used by Wren et al. [13], reported sensitivity and negative predictive values of 100%, with specificity and positive predictive values of 98.4% and 95.3%, respectively. Interestingly, 5 nasal samples showed PCR inhibition. After resolution of discrepant results, PCR detected 3.5% more MRSA than culture on mannitol salt agar. In the study by Huletsky et al. [11], the assay turnaround time was ∼1 h.

When it comes to infection control screening, quick detection of VRE in perirectal samples is a significantly greater challenge for the clinical microbiology laboratory than detection of MRSA in nasal swab samples. There have been several efforts recently to improve detection of VRE using molecular methods. Sloan et al. [14] used a commercial ASR for *vanA* and *vanB*,
coupled with an automated DNA-extraction method, to screen perirectal swabs from 894 patients for VRE. The PCR assay results were compared with growth on agar medium containing 6 or 8 µg/mL of vancomycin. The sensitivity and specificity of the assay were 100% and 97%, and the positive and negative predictive values were 42% and 100%, respectively. It required 3.5 h to process 32 samples, compared with 3–5 days for the traditional culture-based assays. The number of samples with positive PCR results was small (25 using agar containing 6 µg/mL of vancomycin) but illuminated a critical issue regarding discrepancies between PCR results and the results of culture. There was a high correlation of detection of vanA via PCR and recovery of VRE isolates harboring a vanA determinant. However, of 34 discrepancies in which the PCR assay had positive results and the culture had negative results, 29 were due to PCR detection of vanB sequences. There are a series of reports in the literature describing the presence of vanB sequences in anaerobic bacteria, such as *Clostridium butyricum*, and other anaerobes [15, 16]. Thus, it is possible that the vanB determinants detected by PCR in specimens that were negative for VRE by culture were, in fact, present in anaerobic organisms. This is likely to remain a significant issue until a method for linking vanB to an enterococcal-specific sequence is reported, similar to linking mecA to orfX in *S. aureus*.

**IMPROVED DETECTION OF ANTIMICROBIAL RESISTANCE IN M. TUBERCULOSIS USING PYROSEQUENCING**

Another emerging technology that is particularly useful for detecting single-nucleotide polymorphisms (SNPs) that are associated with antimicrobial resistance or bacterial strain identification is pyrosequencing [17]. This technology starts with a single-stranded PCR product to which a nucleotide primer is added to initiate a DNA-sequencing reaction. Light is emitted as each complementary base is added by the polymerase to the newly double-stranded product (figure 2). Pyrosequencing assays can be incorporated into 96-well plates, and multiple targets can be assayed in the same 96-well plate, which gives the laboratory tremendous flexibility in designing assays for multiple infectious agents or multiple targets in the same microorganism. Applications of this technology are highly diverse and include bacterial and viral genotyping [17], recognition of drug-resistance mutations in HIV [18], and characterization of linezolid-resistant enterococci [19].

Infection control issues surrounding MRSA and VRE cause continuous but controllable headaches for most hospital epidemiologists, but a potential outbreak of multidrug-resistant *M. tuberculosis* (MDR-TB) in a hospital can raise the anxiety levels of the hospital epidemiologist considerably higher than migraine level [20]. In the United States, it is sometimes possible to forget that one-third of the world’s population is infected with *M. tuberculosis* [21]. Although physicians in the United States have had to cope with MDR-TB for a number of years (particularly in New York, Miami, and Los Angeles [22]), some MDR-TB strains have now become resistant to second-line agents, such as aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserine, and para-aminosalicylic acid. Recent reports from the United States, Latvia, and South Korea show that these extensively drug-resistant TB cases account for 4%, 19%, and 15% of MDR-TB cases, respectively [23]. Thus, rapid methods for determining the presence of MDR-TB and extremely drug-resistant TB are becoming critically important. Fortunately, novel pyrosequencing assays offer a rapid method of assessing mycobacterial species identification, drug resistance, and even *M. tuberculosis* genotype information, all in a single-assay format.

Studies reported by Arnold et al. [24] show that, among 99 *M. tuberculosis* isolates tested, 68% of the isoniazid-resistant isolates had changes in the *katG* locus at position 315, and 92% of rifampin-resistant strains had mutations in the *rpoB* locus, either at position 516, 526, or 531. These investigators identified SNPs associated with several different species of mycobacteria, and they even identified SNPs that allowed differentiation of genotypes I, II, and III of *M. tuberculosis*. It took <6 h to analyze the results of 96 isolates. Further studies by Zhao et al. [25] demonstrated that pyrosequencing reactions to identify *M. tuberculosis* could be performed on material taken directly from mycobacterial culture vials that were positive for growth by acid-fast staining. In <2 h after DNA extraction, 75% of isoniazid-resistant strains and 86.4% of rifampin-resistant strains were identified using pyrosequencing. In addition, 31.3% of ethambutol-resistant strains contained novel SNPs associated with antimicrobial resistance.

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**Figure 2.** Schematic diagram of a pyrosequencing reaction, in which light is emitted as each specific base is incorporated into the growing DNA sequence. The light signals are translated into sequence data. ATP, adenosine triphosphate; (d)XMP, deoxynucleotide monophosphate; dXTP, deoxyxanthine triphosphate; PPi, inorganic pyrophosphate.
with antimicrobial resistance. Although the results of these assays demonstrate that other mutations associated with antimicrobial resistance have yet to be discovered, the rapid turnaround time of the assays and their positive impact on aiding infection control decisions regarding patient isolation and management are encouraging.

LIMITATIONS TO MOLECULAR DETECTION METHODS

The major limitation of molecular assays designed to detect antimicrobial-resistance genes or mutations associated with antimicrobial resistance is that one can only test for what is already known. As noted with both of the mycobacterial pyrosequencing studies, other mutations associated with isoniazid, rifampin, and ethambutol resistance await discovery. Although a positive test result (i.e., detection of a mutation) correlated highly with antimicrobial resistance in these studies [24, 25], a negative test result has a much lower predictive value.

CONCLUSIONS

The use of molecular assays for detecting MRSA and VRE directly in clinical samples is increasing, in part because of the availability of FDA-cleared kits and ASRs for mecA and vanA/ B, respectively. However, the enthusiasm for broader implementation of molecular methods may be dampened slightly by the limited number of laboratories that can offer assays beyond those that are FDA-cleared. Nonetheless, reports of pyrosequencing assays for M. tuberculosis offer us proof of principle that this novel SNP detection method will positively impact our ability to detect MDR-TB and extremely drug-resistant TB strains rapidly and accurately in the future. Studies showing the cost effectiveness of implementing these molecular strategies as part of ongoing infection control programs are starting to appear, but further studies are anxiously awaited. In much the same way that Maverick finally let go of the memory of his buddy, “Goose,” in the movie Top Gun (throwing Goose’s dog tags out into the ocean in a symbolic gesture), so too must clinical microbiologists begin to replace the slow, traditional culture methods with more rapid and robust molecular strategies. We all feel the need . . . the need for speed.

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References


