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LEARNING OBJECTIVES

Upon completion of this article, the reader will be able to:

1. Understand the study of the relationship between genes and drugs as it applies to individualized medicine.
2. Discuss issues preventing pharmacogenetics testing from being a routine laboratory test.
3. Understand the complexity of pharmacogenetic testing and result interpretation.
4. Discuss the use of or lack of testing standards.
5. Discuss the use of standards in genetic testing.
6. Compare and contrast the development of genetic testing standards in Europe and the United States.
7. Discuss the Gram-stain utilization in the laboratory.
8. Discuss the PNA-FISH assay.

The laboratory's role in pharmacogenetic testing

By Curtis Oleschuck, PhD

Pharmacogenetics, or the study of the relationship between genes and drugs as it applies to individualized medicine, holds great promise for improving patient care. As the interest in translating pharmacogenetic research to clinical practice continues to expand, it is critical that laboratories understand their role in pharmacogenetic testing. Similar to other laboratory tests, pharmacogenetic testing must be properly evaluated prior to being made available. A good analogy made by Dr. David Ransohoff and taken from Bernard Forsher's 1963 article in *Science* titled "Chaos in the Brickyard" is brick layering. To paraphrase his analogy, if the evidence (bricks) for a pharmacogenetic test is faulty, the application of this test (the foundation) will invariably also be flawed. We can use genetic signatures of cancer to illustrate this point.

The expense of genetic testing has historically limited and continues to limit the availability of pharmacogenetic testing for more common use.

Genetic signatures of cancers can be used to predict favorable or unfavorable treatment outcomes. They are derived from analyzing the expression of hundreds of genes in the tumors from a subpopulation of patients. Some well-publicized studies found genetic signatures for breast cancer using this approach. In several of these studies, however, the signature was not confirmed using an independent population of patients and, thus, was prone to bias.[1] This issue is being addressed and draft Food and Drug Administration (FDA) guidelines for the development of technologies related to genetic signatures now include validation using independent populations (see guidelines named on page 15).

TPMT testing

There are several well-studied examples of enzymes whose genetic polymorphisms affect a patient's ability to metabolize a drug. Few clinical laboratories, however, are offering pharmacogenetic tests for these genetic polymorphisms; the exception being thiopurine methyl transferase (TPMT) testing. Many laboratories now offer this test as a preventive measure for patients receiving azathioprine or 6-mercaptopurine. TPMT inactivates both of these drugs, and 0.33% of Western populations have a polymorphism that results in low/absent activity. These individuals are consequently at high risk for drug-induced leukopenia. By measuring either red blood cell TPMT enzymatic activity or TPMT genotyping prior to administering azathioprine or 6-mercaptopurine, a practitioner can determine the probability of a patient developing life-threatening drug-induced leukopenia. For this reason, there is a clear benefit to laboratories offering TPMT testing.

The results of a pharmacogenetic test have the potential to overwhelm practitioners with information.

The FDA has recently approved a blue-label indication alerting patients of CYP2C9 and VKOR1 polymorphisms and their effect on warfarin metabolism. There is a definite relationship between CYP2C9 and VKOR1 polymorphism and warfarin-induced bleeding.[2-4] Additionally, several prospective studies determined that 12% to 27% of dosing variability during the initiation phase of warfarin dosing could be explained by CYP2C9 polymorphisms; this is roughly the same percentage (12% to 27%) as all other factors (e.g., diet and drug-drug interactions).[5-8] The induction phase of warfarin dosing is the time that patients are at greatest risk of warfarin-induced bleeding and, thus, there is a clear benefit to genotyping patients for CYP2C9 and VKOR1 polymorphisms. A few clinical laboratories recently began to offer CYP2C9 and VKOR1 polymorphism testing in the United States, however, the large-scale availability of this testing for all patients receiving warfarin is yet to be determined.

The "thousand-dollar genome"

The expense of genetic testing has historically limited and continues to limit the availability of pharmacogenetic testing for more common use. Several projects are currently underway, including the "thousand-dollar genome," to address this expense; and it is anticipated that in the foreseeable future, cost will not be the limiting factor in the availability of pharmacogenetic testing. Once costs are no longer a major restricting factor, a catalogue of valid tests can be made available for the practitioner to order. The affordability of genomic analysis aside, patent rights for each of the various genes and their polymorphisms presents another major cost barrier.

The results of a pharmacogenetic test have the potential to overwhelm practitioners with information. Even single-gene pharmacogenetic analysis can involve analysis of multiple polymorphisms, and the importance of each polymorphism may not be equal. In addition, multigene analysis, such as genetic signatures for cancers, can involve the analysis of hundreds of genes. Thus, there is a real need to distill this information to a format that is meaningful to practitioners. To address this need, several technologies are being developed to provide interpretation of complex multigene testing. The FDA has addressed these technologies by classifying them as *in vitro* diagnostic multivariate index assays, or IVDMIA, and has produced a number of guidance documents to assist the development of these technologies as they are brought forward:

- Pharmacogenetic Tests and Genetic Tests for Heritable Markers;
- Drug-Diagnostic Concept Paper;
- Drug Metabolizing Enzyme Genotyping System — Class II Special Controls Guidance Document;
- Instrumentation for Clinical Multiplex Test Systems — Class II Special Controls Guidance Document; and
- Gene Expression Profiling Test Systems for Breast Cancer Diagnosis — Class II Special Controls Guidance Document.

Conclusion

In summary, pharmacogenetic testing offers exciting opportunities for clinical laboratory testing to have a greater impact on patient care. As the efforts to make drugs more individualized continues beyond the success of HER-2/neu-Herceptin and as this field expands, the laboratory will play a critical role in offering those tests required to guide this approach to treating patients. Charged with this task, we must continue to evaluate the science behind each test. The National Academy of Clinical Biochemistry has drafted Laboratory Medical Practice Guidelines for pharmacogenetic testing (*www.aacc.org*), which will likely expand along with the growth of this new and exciting field.

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Why is there not a *de facto* gold standard for genetic-testing controls?

Where we are now, and where we can go?

By Michael Murphy, MSc

Genetic testing offers the promise of a quantum leap toward the understanding and treatment of a wide array of diseases and conditions — and, ultimately, the effective application of personalized medicine. In the United States, however, the fulfillment of that promise may be delayed by the lack of a clear roadmap for oversight of the standards and controls used to assure assay dependability and accuracy. And in the absence of a "gold standard" — officially sanctioned guidelines for reference controls for *in vitro* diagnostics — genetic-test developers are limited in their ability to innovate with confidence; laboratories are forced to rely on lower-quality materials created for a research environment; and consumers, facing confusing claims and reports of false positives, lack confidence in the tests that are available.

The European approach

In contrast, the European Union (EU) has taken a more systematic and progressive approach to this situation by starting with the establishment of viable standards, which then provide a clear and accepted roadmap for the development of new assays. The EU also recognizes that it is not always possible, or wise, to rely on a single source for the development of these clinical reference controls. Through EuroGentest, a continent-spanning organization funded by EU members, experts determine which areas are in most urgent need of standards development and which corresponding governmental agency is best suited to handle each of these areas. The stated goal is to provide "the harmonization of standards and practice in all these areas throughout the EU and beyond."

The current situation in the United States

Ideally, quality reference controls would provide the gold standard against which all clinical assays could be judged, and would serve as the starting point for the enhancement and development of new genetic tests. Currently, assay developers in the United States are forced to approach the situation backwards: They develop the test and *then* create the standard.

What this means in practice is that all that can be known about the assay is only as much as can be gleaned from the relatively small set of samples used in development. Even more detrimental is the fact that once the assay becomes available for more widespread clinical use, there is no broadly accepted standard against which laboratories can measure accuracy and reproducibility during the testing of actual patient samples.

Aside from the minimal standards established under the Clinical Laboratory Improvement Amendments (CLIA) for validating research-grade materials (intended only for academic-research purposes) the industry is essentially left to police itself — which leaves laboratories facing the question: If an assay is going to be used for clinical purposes, and the government is not taking the lead in providing clinical reference controls to match it against, what are the alternatives?

Limitations for laboratories

Laboratories bear the burden of validating reference-control material or risk being cited for a violation during a CLIA inspection. In any case, given the choice, most labs obviously would choose to practice genetic testing using the highest quality standards. But they face a roadblock to doing this — namely, that there is no readily available source for the appropriate (which is to say, clinical-quality) DNA controls.

In fact, while one arm of the government regulatory apparatus — the Centers for Disease Control and Prevention (CDC) — is actively advancing the use of research-grade controls, another — the Food and Drug Administration (FDA) — has not enforced regulatory control over the marketing of such reference controls that fail to meet even minimal FDA guidelines. This leaves labs with a dilemma: Either use controls obtained without proper ethical consent of the donor, or use controls that are known to be created under lesser quality standards.

Less-than-ideal workarounds

In the first instance, laboratories routinely will retain a blood sample that is left over from patient tests, purify it, and use it as a control for as long as the sample lasts. Since only a small amount of blood is typically needed for any individual assay, one tube can supply enough DNA for anywhere from 2,000 to 10,000 additional tests.

In addition to the fact that this requires the constant recreation of reference "standards" from different patients, this procedure is ethically questionable. There is no informed consent from the patients whose blood samples are used; they are almost certainly unaware that such a use of their bodily fluids is even possible. Nothing in current guidelines specifically addresses or allows this process, but no regulatory agency has stepped in to stop it.

Many laboratories, then, look to the second choice: using a DNA source that is approved for *research*, not *clinical* use. As mentioned above, such controls are readily available and advertised by government agencies, by way of postings at their websites as a viable source of such materials.

Ironically, although it has met a valid research need, this source was never intended for widespread clinical application. Unfortunately, this creates a situation in which the control material is "manufactured" in a way that does not meet the minimum requirements of all the other testing components that go into a typical PCR-based genetic test. What users end up with is something that is actually the complete antithesis of a true gold standard.

Where do we go from here?

Given the current regulatory landscape and financial implications, there is no incentive for the laboratory industry to make the switch from research-grade to clinical-grade material on its own. CLIA allows for the use of research-grade reference controls, leaving up in the air the entire question of when should only FDA-cleared products be used. Plus, it is more cost effective for labs to keep doing what they have been doing — especially given the fact that, unlike the assays themselves, insurance providers do not currently reimburse the cost of the control material.

This unfortunate situation exists despite the fact that everyone agrees on the need for up-to-date standards that explicitly address clinical reference controls. Such standards would provide a range of benefits:

- For assay developers, government regulation can be a driver of innovation — within a defined regulatory framework, companies know what is permitted and endorsed, so they have a clear playing field for development.
- For laboratories, established standards offer improved consistency, reliability and quality — not to mention fewer false positives and negatives.
- For the general public, concrete guidelines help establish a level of trust and confidence that is necessary to overcome any initial misgivings about genetic testing.

Those in the genetic-testing industry cannot afford to stumble out of the gate with unreliable, inconsistent tests. Based on the European example, it is possible for a governmental agency (even one operating across international borders) to develop sensible standards that provide a clear roadmap for the creation of new assays, and instill a high level of confidence that clinical applications are being judged against a true gold standard.

It is time for government to play a similar leadership role in the United States, so that the promise of genetic testing can more effectively be fulfilled.

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The Gram stain goes molecular

By Philip Onigman and Joe Romano

Every year, 350,000 patients in the United States contract bloodstream infections, causing over 90,000 unnecessary deaths and significant costs to our healthcare system. The mainstream early diagnostic process continues to revolve around the Gram stain, which is performed in laboratories to provide diagnostic feedback to support strategies for patient treatments.[1] Unfortunately, the Gram stain, invented in 1884, is now 124 years old. Physicians still rely on basic Gram-stain results for the first 24 to 48 hours of antibiotic strategy for hospital patients who show a positive blood culture. To compound the issue, we are now over 60 years into the era of antibiotic usage, which is now largely characterized by rampant antibiotic resistance.

Before antibiotic resistance, it could be argued that the Gram stain actually served clinicians, physicians, and patients well, offering the first reliable initial guidepost on the road to antibiotic choice. "Is it Gram positive or Gram negative?" is the first question of the physician to the lab after a positive blood culture is reported. The genesis of antibiotic resistance started at the crossroads between when the diagnostic test was ordered and initial treatment, which often translates into providing the patient with "broad coverage."

Broad coverage refers to the practice of doctors prescribing antibiotics when they believe there is some type of infection — although they have not determined what the infection is (this practice is also known as "empiric therapy"). *Broad coverage and related practices are no longer working* — and one only needs to look at the brief history of antibiotics to understand why. Hospitals tend to be somewhat universal in their use of antibiotics when it comes to coverage for Gram-positive infections.[2] If you asked a physician in the 1960s or 1970s what his antibiotic of choice was, it was usually penicillin. Later, it became ampicillin or methicillin. Currently, it is vancomycin, a

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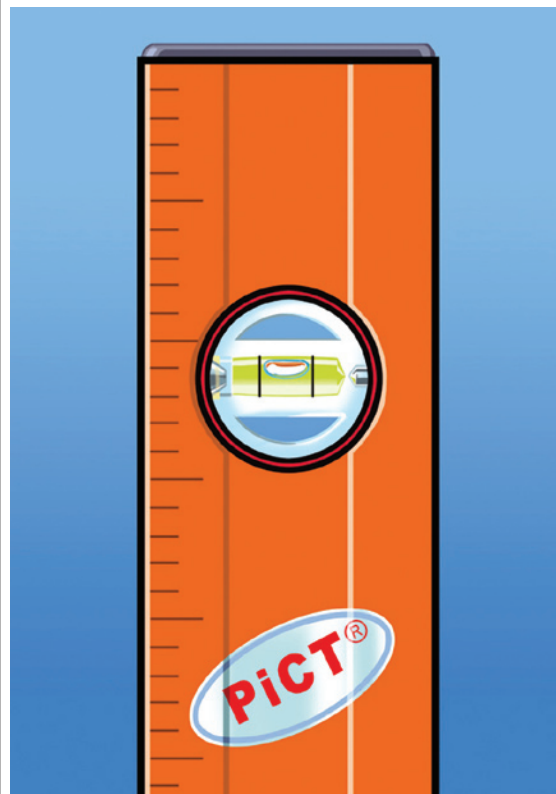

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drug invented in the 1950s that was, for many decades, held in reserve as the drug of last resort. The penicillins were relatively non-toxic, had a wide therapeutic index, and — as generics — were considered inexpensive enough to be used without restriction. Therefore, any physician could traditionally prescribe a coverage drug for a Gram positive, while waiting for species identification. Unfortunately, this has led to the excess use of antibiotics over the years. The habit of broad-based coverage is not new. The selection of the coverage drug is based on individual hospital guidelines.

Rapid tests can speed lifesaving intervention

As a result, over 70% of bacteria that cause infections are now resistant to at least one of the drugs most commonly used to treat them,[3] according to the CDC. Moreover, using traditional diagnostic methods, 48 hours to 72 hours might elapse before physicians know exactly what a suspected infection is. In most cases, they will proceed to prescribe antibiotics anyway, based on the rationale that if there is a true *Staphylococcus aureus* infection and if the doctor waits too long, then there is the potential for a runaway infection. Traditionally, at the first sign of an infection, physicians usually treat patients empirically with broad-spectrum antibiotics. To complicate issues in the laboratory, over 30% of positive blood cultures are due to contamination with coagulase-negative Staphylococci (CNS) that are commonly found on the skin.[4] Because, however, these bacteria are closely related to the more dangerous *S aureus*, and look identical with the Gram stain, patients with CNS-contaminated blood cultures are often given antibiotics such as vancomycin for several days in an attempt to target any possible bacterial strains present.

If the bacteria turns out *not* to be *S aureus*, however, then the physician may have overprescribed for an infection, and if it *is* in fact *S aureus*, then the conventional antibiotic dosage that is prescribed may not be strong enough to kill the infection and could likely fuel resistance. Simply put, *S aureus*-positive blood cultures require aggressive intervention — and simple coverage with vancomycin is no longer considered to be completely effective. Moreover, *S aureus* necessitates the need for physicians to dose vancomycin right to the maximum borderline-toxicity level (15 µg/mL to 20 µg/mL in serum) to be effective.[5] It is either high-dose vancomycin or the physician needs to campaign with the pharmacy department and infectious disease for permission to use daptomycin, linezolid, or some of the newer approved therapies such as tigecycline.[6] Even with the newer, higher cost drugs, clinical success and optimal patient outcome associated with *S aureus* is not assured. This results not only in rising incidences of resistant strains of bacteria but also means that patients are not getting the targeted treatment that could lead to their more rapid recovery.

One point of agreement is certain: The sooner the physician knows it is *S aureus*, the sooner lifesaving intervention can be initiated.[7] Yet, this intervention cannot be delivered by using conventional processes and approaches. There is a need, therefore, for an accurate and rapid blood-culture test, which can inform physicians about the precise type of infection that a patient has.

The movement toward speed and accuracy

The 1980s brought the advent of instrumentation procedures resulting in the development of synthetic oligonucleotides. These were sophisticated instruments sold to government-funded research labs contributing to many facets of molecular biology, including the Human Genome Project. Bacterial and yeast genomes, smaller and simpler than the human genome, were characterized first. Scientists started planning the preparation of diagnostic kits using these probes in order to target known sequences that were developed as part of the genome projects.

The adaptation of the rapid test can be used by any hospital, without investment in new instrumentation.

In 1992, the capability for synthesizing peptide nucleic acid, or PNA, evolved.[8] A synthetic oligonucleotide was designed. Scientists could create a linear piece of PNA, which has a linear base sequence of interest. Scientists conceptualized the construction of living pieces of oligonucleotide with a fluorescent tag and incubated in bacteria or other microorganisms to identify its strain, thus leading to diagnostics. In a patient suffering from bacterial infection, identifying the particular pathogen species or strain becomes extremely difficult. PNA is an organic polymer made up of individual bases such as adenine (A), guanine (G), cytosine (C), and thymine (T) with a peptide backbone. The PNA molecules operate by biomimicry of the organic reaction pattern of a natural DNA molecule. Being constructed out of adenine, cytosine, guanine, and thymine by itself, PNA probes tend to bind (through hydrogen bonding) to a target gene segment, made up of a complementary linear sequence of A, T, G, or C base pairs, specific to the particular killer strains. As a result, the PNA probe can easily enter the *in-situ* environment of the cell. In the growth phase, bacteria and yeast cells produce copious amounts of ribosomal RNA, or rRNA, structures. These rRNAs are said to contain regions of highly conserved, species-specific sequences, which are, therefore, ideal targets for identification assays.

The PNA probe fragment is tagged/labeled with a fluorescence molecule that produces specific color, and hybridizes *in situ* to the target rRNA fragment belonging to the disease-causing killer microbial strain. This results in a simple, sensitive, and specific hybridization assay suited for rapid and accurate identification of the killer microbe. Simply stated, the organism that is identified can be clearly seen using fluorescence microscopy.[9]

The assay uses fluorescent-labeled PNA to target the species-specific rRNA in microbes (*S aureus*,[10-11] *Candida albicans*,[12-15] *Candida glabrata*,[16] *Enterococcus faecalis*, and other Enterococci[17] including *Enterococcus faecium* [OE]), rendering the target cells fluorescent and easily observed using microscope equipment already present in most laboratories. Thus, the adaptation of the rapid test can be used by any hospital, without investment in new instrumentation. These new diagnostic procedures facilitate the dynamic and rapid testing of the blood sample without the need for the time-consuming repeated culture and cell-typing experimentation. Further, this technology speeds up treatment procedure and prevents the unnecessary use of drugs, thus minimizing the chances of creation of drug-resistant bacterial strains. These assays take about two hours in contrast to traditional methods that may take up to 72 hours, thus aiding attending physicians in charge of patients to make rapid diagnosis and, more importantly, saving patients' lives through early, appropriate, and effective antibiotic

therapy. Going further, this technology can result in significant cost savings and development of new therapeutic guidelines.[18] As a result, rapid diagnostics have become a practical reality that can be easily implemented and used, thereby enabling the hospital to become less reliant on empirical coverage to provide a more targeted antibiotic therapy.

Laboratories that utilize PNA FISH (fluorescence *in-situ* hybridization) results have reported a high degree of physician satisfaction with these programs. Antibiotics and antifungals can be more prudently prescribed, patients are discharged sooner, fewer antibiotics are used, and mortality trends are improving. Speed when combined with accuracy in diagnostics really can work. Having a rapid diagnostic result within an hour[19] is a dramatic improvement compared to 2.5 hours, and has been achieved within only four years. But how does a multisite facility with thousands of physicians, nurses, and pharmacists use rapid-diagnostics information?

Consumer-electronics adaptation is running ahead of ability for most hospitals to fully utilize rapid-information systems for their healthcare workers. Think about how quickly conveniences such as cell phone, mobile e-mail, text messaging, and global-positioning systems, or GPSs, have moved from being a "cool technology" to a downright necessity. As large institutions integrate their patient databases to include lab results, pharmacy history, and patient-chart information, the "complete picture" of the patient will be readily available to the attending physician. Ultimately, within the next several decades, every physician will be able to catch every infection as soon as it is detected, with a specific, tailored therapy for every patient.

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