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From bedside to bench and back

Gary William Moore

INTRODUCTION

An oft-quoted statistic is that 70% of clinical decisions are influenced by laboratory medicine data [1]. Some go further and claim this pertains to critical decisions yet systematic evidence is lacking. It is certainly true that a similar percentage of information in electronic medical records consists of laboratory data [1–3] yet frequency of testing does not automatically equate to a proportional effect on major clinical decisions. That itself pre-supposes clear distinction between what constitutes major and minor decisions and perhaps we should be more concerned about necessary or useful decisions? The main reasons for ordering laboratory tests [4] are given in Table 1 and an important point is that a negative result can be as crucial as a positive one. Some might argue that research does not belong in that list yet results from diagnostic testing are often integral to clinical trial data, and residual tissue can be an invaluable resource [5–7]. Unselected testing is widely practiced. For instance, it is common to perform a preoperative full blood count and clotting screen on patients who are asymptomatic for anemia and hemorrhage and indiscriminate test ordering should be discouraged [8]. The aim of this article was to take a glimpse at the diagnostic journey from bedside to laboratory bench and back, the 'vein to brain' cycle, with a spotlight on the impact of the science of laboratory analysis on clinical decision making.

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Table 1: Decision categories for ordering laboratory tests

Category	Reasoning
Diagnosis	To rule in or exclude a diagnosis in a symptomatic patient
Screening	Screen for disease in asymptomatic patients
Monitoring	Assessment of disease progression/regression Drug monitoring Prognostic information
Research	Increase understanding of pathophysiology

From the bedside...

The skill and experience of the clinician is paramount in deciding the appropriate tests to order. Medicine is often a process of elimination and a battery of tests is often indicated at the initial diagnostic stage, which will inevitably generate some of those valuable negative results. Ubiquitous and rapidly available analytes such as hemoglobin, mean cell volume and blood film appraisal can dictate the diagnostic direction of the patient's other blood samples down routes to detect nutritional anemia, hemoglobinopathies and red cell enzyme or membrane disorders to inform treatment. Thrombotic thrombocytopenic purpura and heparin induced thrombocytopenia are usually clinical diagnoses with laboratory data often providing confirmation after treatment has been initiated. Conversely, hemophilia A and B are clinically indistinguishable and laboratory data indicating whether the deficiency is in factor VIII or factor IX will inform the patient's treatment for the rest of their life. Concerns have been expressed that the practices of history taking and physical examination have increasingly given way to reliance on an array of readily available, objective laboratory data [9, 10]. Ordering an inappropriate test, not ordering an appropriate test or misinterpreting the result of an appropriate test can cause harm to patients, as can delays in getting critical results [10]. Assuming an appropriate test is ordered, we move into the laboratory.

...to the bench

As a medical laboratory scientist, it is both pleasing and concerning that many of the medical staff who use our services implicitly trust that the results we report are definitive. Much as I would like to confirm this is always the case, and it often is, there are myriad issues that impact on diagnostic validity, some being easier to control than others. The list is long and includes issues such as between-test and between-reagent variation, quality assurance procedures, reference interval generation and application, assay clinical performance characteristics, human error, analytical equipment failure and many more [4, 10]. What I aim to concentrate on here is a less widely recognized aspect of laboratory medicine, that of variation between types of test that are apparently measuring the same analyte, mining my own sub-specialty of hemostasis and thrombosis for examples.

At first thought it may be difficult to envisage that a test as basic as a prothrombin time (PT) could be problematic yet we are all aware of the necessity of the international normalized ratio in vitamin K antagonist monitoring [11]. Perhaps more intriguingly, a group of dysfunctional factor (F) VII variants are characterized by normal FVII antigen levels but procoagulant activity that varies with thromboplastin species [12]. The Padua and Tondabayashi variants prolong PTs derived from rabbit thromboplastins but not human or bovine, whilst FVII Padua2 will prolong bovine-derived PTs but not human or rabbit. The Shinjo variant only gives normal results with human-derived reagents whilst FVII Nagoya is the only one that will elevate PT with human-derived reagents, and it is distinguished by greater elevation with rabbit thromboplastins and normal bovine-derived PT. Not all are associated with a bleeding phenotype [13]. A similar situation exists within the spectrum of FX deficiencies where a group of dysreactive variants exists with variable presentation in routine coagulation tests [14]. The textbook presentation of FX deficiency is elevation of PT, activated partial thromboplastin time (APTT) and, if performed, Russell's viper venom time (RVVT). However, FX Friuli does not elevate RVVT, FX Padua commonly elevates only PT and FX Melbourne may only elevate APTT. Although each variant will nonetheless manifest in routine coagulation screening, an isolated elevated PT or APTT will suggest the deficiency resides in a different pathway and at best prolong the diagnostic process, or worst, result in non-performance of critical follow-up factor assays.

A technique/principal variation issue that has received increased attention in recent years is that of measuring FVIII activity levels in non-severe hemophilia A. About one-third of patients with non-severe hemophilia A exhibit a discrepancy of FVIII activity when measured by one-stage coagulation assays or two-stage assays, which can be based on coagulation or chromogenic principles [15]. This phenomenon was first described in patients whose FVIII activity was higher in one-stage than two stage assays, typically double, and subsequently, the 'reverse/inverse discrepancy' was described with higher results in two-stage assays [16]. Interestingly, the discrepancies arise as a result of the amount of time the mutant FVIII spends in the presence of thrombin in each assay. Thrombin is available for longer periods in two-stage assays. Thus, a FVIII that is unstable once activated generates higher levels in one-stage coagulation assays since the relatively short period in which thrombin is available prior to clot generation permits FVIIIa to achieve a degree of FIX co-factor activity before stability is lost. The reverse discrepancy occurs with FVIII molecules that are resistant to thrombin activation, so the prolonged period of thrombin availability in two-stage assays increases FVIII activation, generating a higher two-stage result which can be more than triple the one-stage value [16]. The risk of misdiagnosis is clear and laboratory testing in cases of suspected hemophilia A should employ both types of assay.

A particularly challenging area with respect to assay principle variation is detection of hereditary thrombophilias. Standard antithrombin (AT) activity assays are based on inhibition of an excess of either thrombin or FXa in the presence of heparin. Significant differences between sensitivities of thrombin and FXa-based assays have been described when assaying certain dysfunctional variants [17]. AT Cambridge II and AT Denver, which are reactive site defects, will be detected in thrombin-based assays but generate normal activity levels in FXa-based assays. Bovine thrombin and FXa-based AT assays are sensitive to AT Wobble but assays employing human thrombin tend to generate higher, sometimes normal, values. Prolonged incubation with heparin can overestimate the AT level if a heparin-binding defect (HBD) is present. An assay has been described that generates a ratio between AT activity results from short and prolonged incubation times that is sensitive to many AT HBDs [18]. No other alterations are made to the assay conditions and a reduced heparin-antithrombin binding (HAB) ratio is realized in many AT HBDs but not in patients with quantitative deficiencies or reactive site defects.

Dysfunctional variants of protein C (PC) can also generate discrepant results depending on the type of assay employed to measure biological activity [19]. PC activity assays adopt either clotting or chromogenic-based principles but have the common starting point of PC activation by a fraction of Southern Copperhead (*Agkistrodon contortrix contortrix*) snake venom. The activated PC (APC) can then be challenged to cleave a chromogenic substrate or anticoagulate a coagulation-based test such as APTT or RVVT. Reacting APC with a chromogenic substrate bypasses binding of APC to physiological substrates, cofactors and phospholipid, that is, the anticoagulant activity, and only detects quantitative defects and qualitative defects of the activation site and active site [20]. Clotting-based assays will detect all sub-

types but are less reliable as they are affected by a greater number of variables. The presence of FV Leiden can lead to underestimation of PC activity in clotting assays, as can an elevated FVIII in an APTT-based assay, and presence of therapeutic anticoagulants or lupus anticoagulants (LA) can overestimate. Chromogenic assays are not available for assaying protein S (APC-cofactor) activity and the clotting assays, based on PT, APTT, RVVT or activation by FXa, can all underestimate in the presence of FV Leiden. Elevated FVIII can underestimate in APTT-based assays and LAs variably interfere. Differences in results obtained for protein S deficient subjects have been reported with different protein S activity kits [21, 22]. The interesting thing here is that presence of one thrombophilia can interfere with detection of another and teasing apart the possibilities of various assay interdependencies is an invaluable skill. Dilution of test plasma in plasma deficient in the analyte being measured can reduce or eliminate interferences in PC and protein S activity assays but may reduce sensitivity when levels are low [19, 20].

Lupus anticoagulants are a common interference in clotting-based assays seeking to quantify activity of specific coagulation parameters. They are particularly problematic when attempting to accurately identify and separate conditions with similar clinical presentation, such as PC or protein S deficiencies, especially since they can co-exist. Detecting LA themselves is fraught with analytical difficulties due to assay interference by other causes of elevated clotting times [23]. Furthermore, no single assay is sensitive to all LA, which is largely due to antibody heterogeneity and reagent variability. This necessitates using two assays, of different analytical principles, to maximize detection rates. These are normally dilute Russell's viper venom time and a LA-responsive APTT, although other assays such Taipan snake venom time can be useful in certain situations, such as testing anticoagulated patients [23–25].

This is by no means an exhaustive review of the effect of assay principles on detection of hemostatic abnormalities, and neither is it confined to this sub-specialty or even hematology in its entirety. More importantly, it gives a flavor of how knowledge of the principles and practice of the science behind pathology can impact diagnostic decisions and emphasizes the value of cooperation between scientists and clinicians and interpretive reporting.

...and back

Receiving an informative report or direct liaison between laboratory and clinical teams maintains a valuable scientist–clinician interface with potential to enhance diagnostic outcomes. Although many of the instances given above are rare, situations where standard assays do not reflect clinical phenotype can prompt application of alternative measurement techniques to achieve otherwise elusive diagnoses.

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