EDUCATIONAL COMMENTARY – OVERVIEW OF COAGULATION TESTING

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Learning Outcomes

On completion of this exercise, the participant should be able to

- differentiate between primary and secondary hemostasis;
- recall the steps in the coagulation cascade, including the intrinsic, extrinsic, and common pathways;
- list and describe the usual coagulation screening tests;
- describe preanalytic errors that may affect the accuracy of coagulation testing; and
- explain how coagulation tests are used to monitor anticoagulant therapy.

The term hemostasis encompasses the body’s total system to stop abnormal bleeding. Hemostasis is usually divided into primary and secondary hemostasis. Primary hemostasis refers to the actions of the vascular system and platelets, which work together to form an initial plug to stop bleeding after an injury. Secondary hemostasis involves circulating coagulation proteins or factors that work together to form a more permanent fibrin clot. This commentary will review testing for the coagulation system, or secondary hemostasis. The reader should keep in mind that both primary and secondary hemostasis must be considered when testing a bleeding patient.

Preanalytic Variables

Coagulation testing is affected by variations in sample collection, processing, and storage more than any other type of laboratory testing. Studies have shown that the phase of laboratory testing where most laboratory errors occur is the preanalytic. Because coagulation test results may guide diagnosis, treatment, and/or therapeutic monitoring, these errors can have a significant impact on patient outcomes.

Sample Collection

Samples for coagulation testing should be collected using a blue-top tube containing 3.2% buffered sodium citrate as the anticoagulant. The ratio of blood to anticoagulant should be 9:1 if the tube is filled to capacity. “Short draws” (when the tube is only partially filled) result in excess anticoagulant, which neutralizes reagent calcium and may lead to an erroneously prolonged coagulation time. When multiple tubes are collected, the tube for coagulation testing should be collected first or only after tubes with no additives, such as red-top tubes. This is to prevent carryover from tubes with other anticoagulants. According to the Clinical Laboratory Standards Institute (CLSI), it is not necessary to draw a discard tube before the coagulation tube, as was once the standard. The only time this is necessary is when using a
butterfly collection assembly or when drawing from other venous access devices, such as intravenous lines. Needles smaller than 22 gauge can cause hemolysis and should not be used.²

**Processing and Storage**

Samples should be centrifuged at 1500 to 2500g for 15 minutes to prepare platelet-poor plasma (PPP), meaning that there should be fewer than 10,000 platelets/µL in the sample. Each laboratory should establish the appropriate speed and time for its own equipment to achieve this standard. Platelets contain substances that may interfere with various tests if PPP is not prepared correctly. Before centrifugation, tubes should be rocked gently and inspected for clots. Any size clot renders the sample unusable and it must be recollected. After centrifugation, plasma should be inspected for any visible hemolysis, icterus (golden yellow), or lipemia (milky). Hemolysis indicates that the platelets or the coagulation pathway may have been activated, and the sample should be recollected. Icterus or lipemia may interfere with clot detection if the instrument detects a clot optically. Some newer coagulation instruments can compensate for abnormally colored plasma and yield an accurate result. If samples are transported from another facility to the laboratory, care must be taken to avoid elevated temperatures that can enhance degradation of factors V and VIII.¹

Testing is most accurate when performed on fresh samples. If samples cannot be tested immediately, they should be stored upright in a rack with the original stopper in place. Removing the stopper can cause loss of carbon dioxide, which leads to an increase in the sample’s pH that may result in prolongation of prothrombin time (PT) or activated partial thromboplastin time (aPTT). Samples may be stored at room temperature for up to 24 hours for PT and up to 4 hours for aPTT. Samples should not be refrigerated, because this may activate some factors.³ If samples cannot be tested within these time frames, the PPP should be removed and stored at −70°C for 6 months to 1 year.² Frozen plasma should be thawed rapidly at 37°C to prevent denaturation of labile factors I, V, and VIII and tested immediately.¹

**Screening Tests**

The first step in evaluating secondary hemostasis is to perform screening tests. The usual battery includes PT, aPTT, thrombin time (TT), and quantitative fibrinogen test. These are all clot-based tests that rely on the amount of time to reach the end point, a clot. This set of tests is designed to detect most of the abnormalities in the coagulation cascade (Figure 1). The PT test measures factors in the extrinsic and common pathways. The aPTT measures factors in the intrinsic and common pathways. The TT evaluates the very last step in the cascade, conversion of fibrinogen (factor I) to fibrin. If any of these test results is abnormal, more specialized tests are indicated.
Figure 1. Simplified Coagulation Cascade.

**Intrinsic**
Contact activation (PK, HMWK)

- XII → XIIa
- XI → Xla
- IX → IXa + VIIIa, PL + Ca++
- Ca++

**Extrinsic**
Vessel injury TF + VII

- Tissue factor Ca++ + VIIa

**Common**

- X
- Xa
- Xa + Va, PL + Ca++

**Prothrombin**

- Thrombin
- XIII
- XIIIa

**Fibrinogen**

- Fibrin
- Crosslinked Fibrin

Ca++ = calcium, PK = prekallikrein, HMWK = high molecular weight kininogen, PL = platelet phospholipid, TF = tissue factor
EDUCATIONAL COMMENTARY – OVERVIEW OF COAGULATION TESTING (cont.)

Prothrombin Time

The principle of the PT test is to recreate the extrinsic pathway by supplying reagent tissue thromboplastin and buffered calcium chloride. Patient PPP supplies the remainder of ingredients, and the tests times clot formation. The sensitivity of tissue thromboplastin is variable, owing to different sources and preparation methods. Each laboratory must establish a reference range for its reagent and instrument combination.²

The PT test will detect abnormalities in the extrinsic or common pathways of the coagulation cascade (Figure 1). This includes factors VII, V, X, II (prothrombin), and I (fibrinogen). Any suspected single-factor deficiency should be confirmed with a factor assay. The PT test will also be abnormal in acquired conditions such as liver disease, disseminated intravascular coagulation (DIC), or vitamin K deficiency. However, the most common use of the PT test is to monitor anticoagulant therapy.

Activated Partial Thromboplastin Time

The principle of the aPTT test is to recreate the intrinsic pathway of the coagulation cascade (Figure 1). It uses a partial thromboplastin reagent that contains phospholipids to simulate activated platelet surfaces and an activator such as kaolin, celite, or micronized silica to stimulate factor XII to start the reaction. After PPP has been incubated with the aPTT reagent, calcium chloride is added and clot formation is timed.

An abnormal aPTT indicates a deficiency in factors V, X, VIII, IX, XI, or XII, as well as prothrombin or fibrinogen when the fibrinogen level is 100 mg/dL or less.³ As with the PT, a specific factor assay must be performed to confirm a single-factor deficiency. The aPTT is also prolonged if an inhibitor is present, such as anti-factor VIII, anti-factor IX, or other nonspecific inhibitors, such as lupus anticoagulant. A mixing test is often performed following an abnormal aPTT to distinguish between a true factor deficiency and the presence of an inhibitor.

A prolonged aPTT with no clinical signs of bleeding may indicate a deficiency of one of the contact factors, prekallikrein, high-molecular-weight kininogen, or factor XII. These activate the intrinsic pathway in the test tube and interact with the negatively charged activators that are part of the aPTT reagent. It is also important to remember that in some patients a normal aPTT does not exclude the possibility of a mild bleeding disorder and further testing may be warranted.¹

The aPTT may be used to monitor anticoagulant therapy with standard (unfractionated) heparin.
Thrombin Time

The TT recreates the last step in the coagulation cascade, conversion of fibrinogen to a fibrin clot. Thrombin reagent and PPP are incubated separately at 37°C, thrombin is added to PPP, and clot formation is timed. Because it eliminates the need for all other factors and focuses only on this step, this screening test can identify problems with the conversion of fibrinogen to fibrin that may not be detected by PT or aPTT. The TT may be prolonged in cases of hypofibrinogenemia, dysfibrinogenemia, and when heparin, fibrinogen-degradation products, or paraproteins are present. Hypofibrinogenemia may be defined as less than 100 mg/dL fibrinogen (usual reference range, 200-400 mg/dL), and dysfibrinogenemia refers to a biochemically abnormal fibrinogen molecule that is functionally inactive. The reptilase time test is identical to the TT, except that it is not affected by heparin. It is sometimes used to identify the presence of heparin as a cause of a prolonged TT. Reptilase reagent is a thrombinlike enzyme derived from the venom of the *Bothrops atros* viper.

Fibrinogen Assay

This test is very similar to the TT, only it is quantitative. The reference method is the Clauss assay, a clot-based method that uses different dilutions of a known fibrinogen reference plasma or calibrator. A thrombin reagent is added and the time to form a clot is measured for each dilution, similar to the TT. Clotting times are plotted on a chart opposite amounts of fibrinogen, forming a calibration curve. The test is repeated using a 1:10 dilution of patient PPP, and the clotting time is read from the calibration curve. There are other ways to perform this test using immunologic or turbidimetric methods on automated instrumentation for laboratories with higher test volume.

This assay is useful when it is necessary to quantify the amount of fibrinogen present to assess the urgency of bleeding. Acquired disorders such as liver disease, disseminated intravascular coagulation, and primary and secondary fibrinolysis are associated with a decreased fibrinogen level. Dysfibrinogenemia and afibrinogenemia are inherited disorders that show a decreased amount of fibrinogen.

Mixing Studies

The purpose of a mixing study is to differentiate between a true factor deficiency and a circulating inhibitor, such as an anticoagulant or an antibody to a factor. The next step after a prolonged PT and/or aPTT that cannot be explained by known anticoagulant therapy is a mixing study. The prolonged test is repeated using patient plasma mixed with pooled normal plasma (PNP) at several different dilutions. If the prolongation is caused by a true factor deficiency, the PNP will supply factors necessary to correct the test to normal. If an inhibitor is present, the test will remain prolonged, because the inhibitor will continue...
to prolong the test even with PNP. This simple test may prevent the need for more complicated factor assays if an inhibitor is indicated.

**Monitoring Anticoagulant Therapy**

Oral anticoagulants, drugs such as warfarin sodium, are prescribed to prevent stroke in patients with atrial fibrillation and to prevent recurrence of deep-vein thrombosis (DVT) in patients with previous clotting episodes, such as a pulmonary embolism (PE). These drugs are among the most frequently prescribed in North America.\(^4\) The PT test is used to monitor the amount of drug given to a patient. Periodic tests are necessary to monitor dosage, because if the amount of drug is too low, another clot is possible; if the amount is too high, bleeding may occur. Warfarin and similar drugs interfere with the production of vitamin K in the liver and thus affect the vitamin K–dependent factors (FVII, V, IX, and X). Once a patient starts taking the drug, it takes 5 to 7 days before the effects of the drug will be seen in the PT test. The laboratory reports the PT as a calculated value known as the *international normalized ratio* (INR) in an attempt to standardize the test results owing to variations in the strength of the thromboplastin reagent and the instrumentation used in each laboratory. Each manufacturer of thromboplastin reagent has a specific lot number of reagent that is calibrated against a World Health Organization (WHO) standard. The correction factor for their lot number of reagent compared with the WHO standard is called the *international sensitivity index* (ISI). The manufacturer of the reagent supplies a different ISI for that reagent with various instruments that may be used. The closer the ISI is to 1.0, the closer the reagent is to the WHO standard. The ISI supplied by the manufacturer is used along with the PT to calculate the INR for each test. Most laboratories report the PT along with the INR. Most automated instruments calculate the INR and report both values (Figure 2).

**Figure 2.** Formula for calculation of international normalized ratio (INR).

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\text{INR} = R^{\text{ISI}}
\]

\[R = \text{PT ratio obtained with reagent thromboplastin}\]

\[\text{PT ratio} = \frac{\text{patient's PT}}{\text{normal PT}}\]

\[\text{ISI} = \text{International sensitivity index (from manufacturer of thromboplastin reagent)}\]

**Example**

Patient's PT = 25 seconds
Mean normal PT = 13 seconds
ISI = 1.22

\[
\text{INR} = \left( \frac{25}{13} \right)^{1.22} = 2.216 \text{ or } 2.2
\]
The use of the INR enables a standardized therapeutic range (INR, 2.0-3.0) for most patients taking oral anticoagulant drugs. In some settings, such as mechanical valve replacement, the patient may require a level of anticoagulation that exceeds the standardized INR range, as established by his or her physician. Another advantage to using the INR is that by correcting for variations in reagent and instrumentation, it allows better evaluation of long-term anticoagulant therapy no matter where the test is performed.

Another anticoagulant frequently used for therapeutic purposes is heparin. Whereas warfarin is an oral anticoagulant meant for long-term therapy, heparin is used for a short term and is administered intravenously or subcutaneously. Heparin therapy may be followed by warfarin therapy if longer-term treatment is necessary. Heparin may be administered in 2 forms: standard or unfractionated heparin (UFH), and low-molecular-weight heparin (LMWH).

Unfractionated heparin may be prescribed in the following situations: to treat DVT and PE; to provide initial treatment of an acute myocardial infarction; to prevent another obstruction after stent placement; and to maintain vascular patency during cardiopulmonary bypass surgery. Unfractionated heparin is usually administered intravenously for 3 to 5 days for acute situations while the patient is hospitalized. Therapy with UFH has traditionally been monitored with the aPTT test. Each laboratory must establish a therapeutic range for UFH for their aPTT procedure and instrumentation. This is done by collecting 50 samples from patients with different levels of UFH in their plasma. Activated partial thromboplastin times are performed on all samples and compared with the amount of UFH present in each. The paired results are plotted on a dose-response curve. The range in seconds of aPTT results that corresponds exactly to 0.3 to 0.7 heparin units/mL is the therapeutic range. This is known as the ex vivo or Brill-Edwards method of establishing the therapeutic range of UFH. Some aPTT reagents are not sensitive enough to detect heparin at these low levels and cannot be used to monitor UFH therapy.

Low-molecular-weight heparin was developed because of the uncertainty of the dose-response of UFH. It became available for use in anticoagulant therapy in 1993. Therapy with LMWH is being used more often because it has more reliable pharmacokinetics and bioavailability and its dose-response is more predictable than that of UFH. It is usually administered subcutaneously and is often given prophylactically during or after orthopedic surgery, general surgery, or trauma. It may also be used to treat DVT, PE, or unstable angina. If patients taking warfarin require surgery, the warfarin is often discontinued for up to a week before the procedure and replaced with LMWH. Low-molecular-weight heparin does not require monitoring in adult patients except in special cases, such as pregnant patients who have a history of spontaneous abortion, morbidly obese patients, severely underweight persons, or those with renal issues. The anti-factor Xa assay is recommended if monitoring is necessary, because the aPTT is not sensitive to the effects of LMWH.
The anti-factor Xa assay is a chromogenic assay and may be used to monitor both types of heparin therapy. Excess activated factor X (FXa) is incubated with patient PPP. Heparin along with antithrombin present in the patient plasma inhibits the FXa. A chromogenic substrate is added to this mixture and any residual FXa enzymatically cleaves the chromogen, producing a yellow color that is measured spectrophotometrically. Results are compared with a reference curve prepared with known concentrations of heparin and the corresponding absorbance of each. The therapeutic range for heparin is 0.3–0.7 U/mL.4

Conclusion

This commentary has reviewed the basics of coagulation testing. There are many more specialized tests that may be performed in the coagulation laboratory that were not included here.

References


