Laboratory Management of the Bleeding Patient

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ABBREVIATIONS: APTT = activated partial thromboplastin time; DIC = disseminated intravascular coagulation; PT = prothrombin time; PTT = partial thromboplastin time; TCT = thrombin clotting time; vWD = von Willebrand disease; vWF = von Willebrand factor.

INDEX TERMS: Disseminated intravascular coagulation; partial thromboplastin time; platelet count; prothrombin time; von Willebrand disease.

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From the laboratory point of view, test results are only as reliable as the quality of the sample. It is imperative that the specimens received for coagulation testing are collected by a clean venipuncture with as little trauma to the tissues as possible. Clotted, hemolyzed, and contaminated specimens are not suitable and should not be used by the laboratory. The most common causes of erroneous results are related to the blood draw; therefore, special care must be taken to avoid any undue problems. Sodium citrate is the anticoagulant that is most commonly used for both routine and special coagulation testing. The citrated blood should be spun by routine centrifugation methods so that platelet free plasma is obtained.[1]

PROTHROMBIN TIME

The prothrombin time (PT) monitors the factors that are in the extrinsic pathway – factors VII, X, V, and II. A PT is performed by adding synthetically prepared tissue factor, generally from brain or lung, and calcium to the test plasma and measuring the time it takes the clot to form. Prolongation of the PT is most often a result of deficiencies in factor VII but can also be caused by any of the aforementioned factors. Decreased fibrinogen, levels less than 100 mg/dL, will also prolong the PT; however, this test is not used to measure fibrinogen deficiency since most coagulation labs can perform a fibrinogen assay.[1]

ACTIVATED PARTIAL THROMBOPLASTIN TIME

The partial thromboplastin time reagent is a preparation of synthetic phospholipids, particulate activator, and calcium. The APTT, or simply PTT, measures the factors that are designated in the intrinsic pathway – PK, HMWK, factors XII, XI, IX, VIII, X, V, II, and fibrinogen. Deficiencies of PK, HMWK, and factor XII show no bleeding tendencies and are of little clinical consequence.

There are several explanations for a prolonged PTT. The first, and most common, is heparin. Heparin may be present as a result of therapy or as a contaminant when it is used to flush a line. If heparin is suspected, a thrombin clotting time (TCT) can be performed. In this test, thrombin is added to plasma, and the clotting time is measured. The reference range for the TCT is 15 to 22 seconds. If heparin is present, the TCT will generally be prolonged to greater than 60 seconds.

A second possibility for a prolonged PTT is a factor deficiency. Most factor deficiencies are congenital and cause bleeding problems in children. Adult onset factor deficiencies are rather rare but can be the cause of severe bleeding problems. Acquired multiple factor deficiencies will have a PT and PTT of greater than 1.5 times the mean of the reference interval. Some causes of acquired factor deficiencies include liver disease, vitamin K deficiency, and drug therapy. To distinguish between liver disease and vitamin K deficiency, it is best to use factor V and factor VII levels. In liver disease both factors will be decreased, but in Vitamin K deficiency only factor VII will be low.

Finally, the presence of an inhibitor can also cause an abnormal PTT result. Inhibitors can be non-specific such as a phospholipid dependent inhibitor like lupus anticoagulants,

or they can be directed against a specific factor. Specific factor inhibitors, such as VIII or IX, prolong the PTT by lowering the amount of factor present.

Mixing studies should be performed to differentiate between a factor deficiency and the presence of an inhibitor. In this test, pooled normal plasma is mixed with the patient's plasma in equal volumes, and a PTT is run on the sample. If the result is within five seconds of the pooled normal plasma, the mix is said to have corrected, and a factor deficiency is suspected. If there is no correction, the possibility of an inhibitor should be investigated.[1]

PLATELET COUNT

At any age, a normal platelet count is 150 x 10^9/L to 450 x 10^9L. Whenever the platelet count drops below 150 x 10^9/L, termed thrombocytopenia, it should be confirmed by examining a blood smear. The peripheral blood smear is important because the automated cell counters that are used in most laboratories can over or underestimate the platelet count. Refer to Table 1 for a list of possible causes of interference with the automated platelet count.

DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation (DIC) is a condition in which there is a generalized activation of hemostasis. In this condition, coagulation factors and control proteins, platelets, and fibrinolytic enzymes are consumed; even though this is a thrombotic process, hemorrhage is often the most obvious symptom.[2] The diagnosis of DIC must be confirmed quickly since acute DIC can be fatal. Clinically, however, DIC can present a very confusing picture. A laboratory panel of a platelet count, a PT, PTT, fibrinogen assay, and D-dimer assay is essential to aid the diagnosis. Expected results for a DIC panel can be found in Table 2.

Table 1. Possible interference with automated platelet counts

False increase
Microspherocytes
Red blood cell and leukocyte fragments
Bacteria
Pappenheimer bodies

False decrease
Poor collection techniques
EDTA–dependent platelet agglutination
Platelet cold agglutinins
Platelet satellitism

The fibrinogen assay is similar to the TCT. In this test, thrombin is added to the sample plasma and acts as a catalyst to convert fibrinogen to fibrin. The two tests differ in that the fibrinogen assay uses diluted plasma at a 1:10 with Owren's buffer, and the thrombin reagent used is at a higher concentration than that used in the TCT assay. These two variations give an "inverse but linear relationship between interval to clot formation and concentration of functional fibrinogen".

The D-dimer assay is essential to the diagnosis of DIC. D-dimers are found in almost all cases of DIC; however, since D-dimer levels can be elevated in other conditions, it is imperative that the other tests in the DIC panel be used in conjunction with the D-dimer assay to reach a diagnosis.

Currently, there are two methods of testing D-dimers. The first is a semi-quantitative method in which latex particles are coated with monoclonal antibodies to D-dimers. Serial dilutions are made of the sample plasma and then mixed with the latex suspension. The test is read manually, and a positive result is determined if agglutination of the latex particles is observed. DIC is indicated by a positive reaction in the 1:2 dilution which suggests D-dimer levels of greater than 500 ng/mL.

The quantitative D-dimer assay is either an enzyme immunoassay or microlatex particle immunoagglutination assay method. The latter test can be automated and produces a result with a relatively rapid turn-around time. The quantitative D-dimer assay is most useful for its negative predictive value to aid in ruling out localized venous thrombosis such as pulmonary emboli and deep vein thrombosis. A normal, healthy adult can be

Table 2. Expected results for a DIC profile

Test	Normal range	DIC
Platelet count	150 – 450 x 10^9/L	<150 x 10^9/L
PT	11 – 14 seconds	>14 seconds
PTT	25 – 35 seconds	>35 seconds
Fibrinogen	200 – 400 mg/ dL	<200 mg/dL
D-Dimer	0 – 240 ng/ mL	>500 ng/mL

expected to have a D-dimer of less than 240 ng/mL. Higher levels are expected in patients with clots but are also seen in many other diseases. Clinical history and the laboratory tests previously described will aid the physician in reaching the correct diagnosis. Table 3 shows the correlation between the semi-quantitative and quantitative D-dimer assays.[3]

FACTOR ASSAYS

Hemophilia A, or factor VIII deficiency, is the most commonly inherited defect of the coagulation factors. The disease is an X-linked chromosome disorder; so therefore, it is primarily expressed in the male population. Laboratory screening tests show a normal PT and TCT. The PTT will generally be prolonged, but only if the factor VIII activity level is below 40%.

The factors that are necessary for clotting in the intrinsic pathway can each be measured by one-stage clotting assays as a modified version of the PTT. The specific factor assays measure the time of clot formation of test plasma when diluted and mixed with specific factor deficient substrate plasma. The result is compared to a corresponding calibration curve specific to the lot of PTT reagent and factor deficient plasma. Specimens are tested at three dilutions. The dilutions need to agree within 10% to assure that no inhibitor is present. A hemophiliac is considered a severe case if the factor VIII level is less than 1%. A factor VIII level of 1% to 5% is called a moderated hemophiliac, and a mild deficiency is diagnosed if the patient has factor VIII levels between 5% to 25%.

Table 3. Correlation between the semi-quantitative and quantitative D-Dimer assays

Slide latex endpoint dilution	Quantitative D-dimer in ng/mL
Negative	Less than 240
Undiluted	240 – 500
1:2	500 – 1000
1:4	1000 – 2000
1:8	2000 – 4000
1:16	4000 – 8000
1:32	8000 – 16,000
1:64	16,000 – 32,000

Chromogenic factor VIII assays are also available to quantitate the factor VIII level. New recombinant, B-domainless FVIII products that make it necessary to be able to accurately measure activity levels are now on the market. Discrepancies are reported between the chromogenic and clot based methods. Clot-based methods recover up to 50% lower activity for these products than the chromogenic assays which can have significant clinical implications for the management of patients with hemophilia A.

Like hemophilia A, hemophilia B, or factor IX deficiency (also known as Christmas disease), is an X-linked chromosome bleeding disorder. The diagnosis is reached by similar methods as hemophilia A. The PT and TCT will be normal, but the PTT may or may not be abnormal, depending on the sensitivity of the reagent being used. Based on laboratory tests and clinical history, a diagnosis can be determined by performing a one-stage clotting assay using factor IX deficient substrate plasma. As with the factor VIII assay, three dilutions are performed which must agree within 10%, to obtain a factor level of the test plasma.[4]

von WILLEBRAND DISEASE

von Willebrand disease (vWD) is the most common hereditary bleeding disorder affecting up to 1% to 2% of the general population. It is a condition in which there is a either a quantitative or qualitative defect in the von Willebrand factor (vWF), a protein that is necessary to platelet adhesion and acts as a carrier protein for factor VIII. Testing for vWD in the laboratory starts with a panel of tests consisting of a factor VIII activity level, a ristocetin cofactor (also known as vWF activity level), and a von Willebrand antigen level. It is important to note that the vWF level varies significantly according to blood groups. Table 4 shows the mean vWF activity associated with the corresponding blood group. In addition to variation in blood types, vWF can also be increased during pregnancy, hemorrhage, acute infection, and strenuous exercise. As a result, patients with a suspicious clinical history should be confirmed with subsequent testing over a period of time.[5]

The ristocetin cofactor activity, or vWF activity, is determined by the agglutination of standardized platelets in the presence of vWF using ristocetin. The platelets play a passive role in the procedure, but it is necessary that the ristocetin dependent receptor be intact. The vWF activity is the most useful assay to diagnose vWD. In this procedure, platelets are treated with ristocetin in the presence of dilutions of standard plasma using a platelet aggregometer. The ristocetin cofactor activity is proportional to the slope of the platelet agglutination curve.

Once a standard curve is prepared, patient plasma is the source of vWF, an agglutination pattern can be discerned, and the vWF activity is determined from the standard curve.

The vWF antigen can be determined two ways. The first is the ELISA method. A plate is coated with rabbit anti-human vWF antibodies that bind the vWF to be measured. In the next step, anti-vWF antibodies coupled with peroxidase bind to the rest of the free antigenic determinants of vWF. The bound enzyme peroxidase is revealed by its activity in a predetermined time on the substrate OPD (orthophenylenediamine) in the presence of hydrogen peroxide (H_2O_2). After the reaction is stopped with sulfuric acid (H_2SO_4), the color intensity is directly proportional to the concentration of vWF present. Absorbance is measured at a wavelength of 492 nm. The disadvantages to this test are that it is very time consuming and highly labor intensive.

Another option to determine the vWF antigen level is the immunoturbidimetric method. This is an automated assay in which a beam of light is passed through the sample. The sample consists of patient plasma added to a suspension of microlatex particles that have antibodies attached to them. If the wavelength of light is greater than the diameter of the particles, the light will only be slightly absorbed. When there is vWF antigen present, the particles will clump to form aggregates whose diameters are larger than the wavelength of light; therefore, more light is absorbed. This greater absorption is proportional to the vWF antigen level that is in the sample. Table 5 shows the various subtypes of von Willebrand disease, and the profile results expected.

Table 4. von Willebrand factor mean activity by blood group

Blood group	Mean vWF
O	75%
A	105%
B	117%
AB	123%

Once a diagnosis of von Willebrand disease is made, it is sometimes necessary to order more testing to differentiate between the various subtypes. Assays such as the ristocetin response curve (also called low dose ristocetin induced platelet aggregation) will aid in distinguishing type 2B vWD. Platelets in Type 2B vWD are hyper-responsive to low concentrations of ristocetin. In addition, vWF multimeric analysis by SDS polyacrilamide gel electrophoresis can distinguish between Type 1 and 2 and also Subtypes 2A and 2B.

Diagnosis of the bleeding patient can be a difficult task. Clinical history is vital, but laboratory testing provides key information into the patient's condition. Proper use of this information can save valuable time when the need to assess the situation quickly is critical.[6]

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Table 5. Various subtypes of von Willebrand disease and expected laboratory test results

vWD type	vWf activity	vWf antigen activity	Factor VIII activity
1	40 – 60%	40 – 60%	40 – 60%
3	<1%	<1%	<1%
2A	30 – 50%	70 – 150%	70 – 150%
2B	30 – 50%	70 – 150%	70 – 150%
2N	70 – 150%	70 – 150%	40 – 60%
2M	40 – 60%	40 – 60%	70 – 150%