The Laboratory Approach to Inherited and Acquired Coagulation Factor Deficiencies

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KEYWORDS

• Hemophilia • Coagulation factor deficiency
• Hypoprothrombinemia • Hemostasis • Prothrombin time
• Activated partial thromboplastin time • Pre-analytical variables
• Bleeding • Coagulation factor inhibitors

Coagulation factors are usually either enzymes (eg, F9, F2) or cofactors (eg, F5, F8) that circulate at varying concentrations in the blood. Rather than reporting the absolute concentration of the circulating factors (as is done with most chemistry analytes), the clinical laboratory reports *activities* for most coagulation factors (ie, the functional activity of the factor in the patient's plasma compared with that of calibrator or a standard plasma, the latter with a defined assayed activity of 100%).

Factor deficiencies traditionally are classified as type 1 or type 2. In type 1 deficiency, the protein structure of the factor is normal, but there is a decreased concentration in the circulation, so the activity level is decreased. This can be caused by increased clearance or decreased production of the factor. Essentially all acquired and many inherited disorders fall into this category. In type 2 deficiencies, there is a normal or nearly normal concentration of a circulating protein that is intrinsically defective and therefore also demonstrates a lower overall activity level. Either type

This work was supported in part by a grant from Blood Systems Inc.

Note: Although Roman numerals traditionally are used to identify coagulation factors (eg, FIX, FVII), Arabic numerals will be used in this discussion (eg, F9, F7) as the latter are less confusing for the learner and also for an international audience.

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Clin Lab Med ■ (2009) ■–■
doi:10.1016/j.cll.2009.04.002 labmed.theclinics.com
0272-2712/09/$ – see front matter

of deficiency will show decreased activity when measured by laboratory assays. When measuring the protein itself using an antigen assay, type 2 deficiencies show a relative preservation of the antigen level in relation to the activity level, while type 1 deficiency also shows decreased antigen. This situation is illustrated best in von Willebrand disease (vWD) (see article by Torres in this issue).

A brief survey of the clinical aspects of the inherited and acquired factor deficiencies is presented first, followed by a discussion of the laboratory considerations in evaluating patients who have possible factor deficiencies. **Table 1** and **Box 1** illustrate the etiologies, relative incidence, and other characteristics of the various factor deficiencies discussed. Some deficiencies of proteins involved in hemostatic regulation also will lead to bleeding diatheses (eg, alpha-2-antiplasmin); however, these abnormalities are not within the scope of this article.

INHERITED FACTOR DEFICIENCIES

Inherited bleeding disorders have been recognized since ancient times, often caused by severe bleeding with circumcision or minor trauma. Severe hemophilia A and B, and severe F11 deficiency typically prolong the patient's activated partial thromboplastin time (aPTT), but this abnormality is not always overwhelming. Many patients present with an aPTT between the upper end of the normal reference range and the middle of the typical aPTT heparin reference range. Deficiencies of the contact factors—most commonly F12—can prolong the aPTT more significantly, similar to what is seen with a high level of therapeutic heparin or heparin contamination after blood is drawn through a heparinized line. Hemophilia A (F8 deficiency) and B (F9 deficiency) are the only factor deficiencies typically inherited in a sex-linked pattern.

Sufficient hemostasis often can be achieved despite having coagulation factor levels below the normal laboratory reference ranges. Similarly, a modest increase in the endogenous circulating factor activity level (eg, from 0.5% to 5%) in hemophilia can have major clinical implications, essentially converting a severe to a mild phenotype. This provides hope to researchers working to use gene therapy as a possible treatment of inherited hemophilia.[1]

Factor 8 Deficiency (Hemophilia A)

Hemophilia A[2] is the most common inherited factor deficiency. This X-linked disorder is caused by a deficiency of F8. F8 is the cofactor that works with F9 to activate F10, increasing the reaction rate by several orders of magnitude. Older nomenclature calls the F8 *coagulant* activity "VIII:C" and the measurement of the *antigen* "VIII:Ag." The F8 molecule usually circulates in the bloodstream, protected from degradation by forming a stable complex with von Willebrand factor (vWF). Deficiency of vWF leads to increased clearance of F8 from the circulation; therefore, F8 activity typically is included in screening panels for vWD.

Many different genetic mutations have been classified in various hemophilia A pedigrees.[3,4] Mild and moderate hemophilias typically are caused by one of many missense mutations induced by single base pair substitutions. Severe hemophilia A, by contrast, is caused more often by larger gene defects, including inversions (intron 22), large deletions, and insertions. Thus, both activity and antigenic protein measures are typically very low. Intrachromosomal intron–exon recombinations of the X chromosome account for about half of the severe forms. For the prenatal genetic diagnosis of known males to be effective, family studies must identify the mutation particular to that pedigree. There is a high sporadic occurrence (high spontaneous mutation rate), however, of hemophilia also; thus, about 30% of newly diagnosed severe hemophilia

Table 1
Congenital factor deficiencies

Factor Deficiency	Estimated Incidence	Inheritance Pattern/Genes Involved	Bleeding Severity
F8 (hemophilia A)	1:10,000	X-linked recessive (Xq28)	Mild >5% Moderate (1% to 5%) Severe (<1%)
F9 (hemophilia B)	1:30,000	X-linked recessive (Xq27)	Mild to severe
F11	Rare, 5% in Ashkenazi Jews	Autosomal recessive (4q32q3)	Mild to severe
F2 (prothrombin)	Rare	Autosomal recessive (11p11–q12)	Mild to moderate
F5	1:1 million	Autosomal recessive (1q21–q25)	Mild to moderate
F7	1:500,000	Autosomal recessive (13q34)	Mild to severe
F10	1:500,000	Autosomal recessive (13q34)	Mild to severe
F12	Rare	Autosomal recessive 5q33	No bleeding
Other contact factors (including prekallikrein, high molecular weight kininogen)	Rare, unknown	Autosomal recessive (various genes)	No bleeding
F13	<1:1 million	A subunit: 6p24-p25 B subunit: 1q31-q32	Moderate to severe; postoperative
Afibrinogenemia	Rare	Autosomal dominant (various mutations at 4q31)	Variable
Dysfibrinogenemia	Rare	Autosomal dominant (various mutations at 4q31)	Variable—may be asymptomatic
Hypofibrinogenemia	Rare	Autosomal dominant (various mutations at 4q31)	Variable
Combined F5 and F8	Rare	Autosomal recessive (18q21, 2p21)	Variable
Combined F2, F7, F9, & F10	Rare	—	Variable

Box 1
Acquired factor deficiencies

Immune-mediated factor deficiencies

Alloantibody factor inhibitors in hereditary hemophilia patients

- F8 inhibitors in hemophilia A patients on factor replacement therapy (incidence = 24% of hemophilia A patients)
- F9 inhibitors in hemophilia B patients on factor replacement therapy (incidence = 1.5–3% of hemophilia B patients)

Autoantibodies causing acquired hemophilia

- Acquired hemophilia A (most common, may cause severe bleeding)
- Prothrombin deficiency complicating the antiphospholipid syndrome (rare)
- Acquired F5 deficiency (from antibodies to bovine thrombin preparation used in certain fibrin glues, uncommon)

Nonimmune-mediated

Increased destruction

- Disseminated intravascular coagulation (DIC)
- Extracorporeal membrane oxygenation (ECMO)
- Fibrinolytic drugs in thrombolytic therapy (eg, tissue plasminogen activator)

Decreased production

- Liver disease

Abnormal production

- Warfarin use
- Other causes of vitamin K deficiency

Loss and sequestration

- Nephrotic syndrome
- Acquired F10 deficiency associated with light chain amyloidosis (accelerated removal of F10 caused by adsorption of the factor to the amyloid fibrils); 8.7% of amyloidosis patients had F10 levels less than 50% of normal; 56% of these had bleeding complications if F10 less 25% of normal; can be fatal
- Plasma exchange apheresis (particularly decreased fibrinogen)

Inactivation

- Direct thrombin inhibitor use (eg, hirudin, argatroban)

patients may not have a family history. Activity assays sometimes are performed on cord blood from male newborns delivered to a known carrier in a family with severe hemophilia. This is of particular interest in premature infants, as there is increased risk of intracranial bleeding.[5] It is an important caveat that such results must be interpreted with caution, as cord blood is not an optimal specimen for coagulation testing. This is because activation of clotting in cord blood often occurs during the delivery and subsequent blood collection processes, and cord blood samples are clotted commonly, at least partially. In addition, maturation of the hemostatic system occurs during childhood, such that newborns generally have lower levels of some factors than

adults. For example, the mean F9 in newborns has been reported at only 53% for full-term and 35% for healthy premature infants, with F11 at 38% and 30%, respectively. Exceptions to this maturation are F8 and vWF levels, which are typically greater than or equal to100% in both full-term and healthy premature infants.[6] Testing of boys from families that have milder forms of the disease typically can be performed later in childhood, preferably before they begin learning to walk.

The clinical manifestations of hemophilia A usually correlate with the severity of the deficiency measured in the laboratory. Because the difference between severe (less than 1% F8 activity) and moderate hemophilia (1 to 5%) is clinically significant, laboratory assays should have linearity down to less than 1%. In recent decades, there has been dramatic improvement in long-term treatment using both plasma-derived factor concentrates and recombinant factors. Today, because of prophylactic regimens and early treatment of bleeding episodes, many young adult males who have severe hemophilia A do not exhibit some of the classical clinical manifestations that include joint arthropathy from repeated bleeding into joints, contractures of musculature from frequent muscle and joint involvement, hemorrhage from mild trauma, and even central nervous system (CNS) bleeds.

Severe cases often receive prophylactic factor administration, either primary prophylaxis, before development of any joint bleeding (a hallmark of hemophilia), or secondary prophylaxis, after a significant bleed into a vital organ, or after development of a target joint. The US Centers for Disease Control and Prevention definition of a target joint is that a minimum of four bleeds must have occurred in that joint within a consecutive 6-month period. Cryoprecipitate and fresh-frozen plasma revolutionized the treatment of hemophilia A about 50 years ago. The ease of use and safety of purified factor concentrates and their recombinant counterparts, however, have replaced these blood products for hemophilia therapy in industrialized nations. Cryoprecipitate and plasma, however, remain the mainstays of treatment for bleeding in economically challenged countries around the world.

Infusion of factor at home has also revolutionized management of these patients. Home treatment allows for early institution of replacement products at the first sign of a bleed, which decreases the morbidity of these bleeds significantly. In patients who have mild deficiency, DDAVP can be used as an alternative to factor, either intravenously or by nasal spray. DDAVP increases both F8 and vWF and thus aids hemostasis in those hemophiliacs who are mildly affected. However, before its use, each patient should be evaluated for their response to DDAVP. In addition, antifibrinolytic agents can be used as adjunct therapies, especially for mucosal bleeding.

Female carriers of hemophilia typically have low-normal to moderately decreased factor levels, but have been reported as low as 5%. As many as 10% of carriers will have levels less than 30%. Some carriers, therefore, may have a mild-to-severe bleeding phenotype, particularly with major hemostatic challenges such as surgery, and their factor levels will need to be monitored for such procedures.

In addition to using factor activity assays to diagnose deficiency, a significant part of the coagulation laboratory workload is to monitor hemophilia treatment and to screen for increased clearance of administered factor caused by possible inhibitor formation.

Factor 9 Deficiency (Hemophilia B)

Sometimes known as Christmas disease (after the surname of the first patient diagnosed with this disorder), hemophilia B is caused by a deficiency of F9, an enzyme rather than a cofactor, which activates F10. Hemophilia B is analogous to hemophilia A in many aspects. It is only about one-fifth as common, but follows similar clinical patterns in disease manifestation and severity. Although also X-linked, hemophilia B

shows tremendous heterogeneity at the molecular level, making genetic screening much more difficult. The F9 mutation unique to each family needs to be identified before prenatal or other genetic screening can be performed.

Cryoprecipitate does not contain significant amounts of F9 and therefore cannot be used to treat this disease. Prothrombin complex concentrates once were used to treat the disease before specific factor products became available (both plasma-based and recombinant). It is important to note that some patients who have hemophilia B will develop allergic reactions to the infusion of these factors, including anaphylaxis. Hence, the first 10 to 15 infusions usually are administered in a clinic or hospital setting. As in hemophilia A, home therapy is an effective strategy in reducing the morbidity of bleeding in these patients. Patients who have mild hemophilia B may have normal or near-normal aPTT results. Therefore, specific factor assays should be performed in working up undiagnosed mild bleeding disorders, even if the aPTT is within the normal range.

Factor 11 Deficiency

In contrast to hemophilias A and B, F11 deficiency (which used to be termed hemophilia C)[7,8] is an autosomal disorder with variable penetrance. The gene is located on chromosome 4 (4q35.2). In addition, the bleeding manifestations are heterogeneous in relation to the factor activity levels (ie, there is a poor correlation between bleeding manifestations and the baseline F11 clotting activity, unlike hemophilia A and B). Thus, separation of patients into distinct clinical phenotypes is less clear-cut than in other bleeding disorders. Many patients do not exhibit bleeding until challenged with trauma or surgery; unlike hemophilia A and B, bleeding in F11 deficiency is often mucosal. Patients who have severe deficiency (levels less than 15% to 20%) have a high probability of postoperative bleeding. This, however, is not a uniform finding, and some may not bleed at all. Yet, patients who have mild deficiency may have severe bleeding when hemostatically challenged (eg, by platelet-inhibitory drugs). The reason for this discrepancy is unclear, but one possible explanation may be the coexistence of other bleeding disorders like vWD, which could contribute to the bleeding risk in these mildly deficient patients. Hence, it is important from a laboratory standpoint to consider testing for coinheritance of other bleeding disorders. Because many of these patients are asymptomatic until challenged by surgery or trauma, the diagnosis often is made in late childhood or early adulthood. Over half of the cases are diagnosed in patients of Ashkenazi Jewish descent. In this group, it is estimated that one in eight individuals are heterozygous for a gene defect, and 1 in 190 are homozygous.

Generally, screening coagulation tests will reveal an isolated prolonged aPTT with F11 deficiency. Because different partial thromboplastin reagents vary in sensitivity to F11, reference ranges should be established in each local laboratory. F11 assays should be performed based on clinical suspicion. Most identified mutations cause type 1 deficiency, with reduced amounts of normal F11 antigen leading to a lower concentration of the protein in the plasma. Because F11 antigen measurements correlate with activity levels, determination of antigen levels is not recommended routinely as part of the testing process. Given the poor correlation of F11 activity levels and bleeding risk, researchers are looking into global tests of hemostasis (thrombin generation, thromboelastography) to understand the role of F11 in hemostasis (see article by Teruya in this issue). Specific F11 replacement concentrates are available in the European Union and experimentally in the United States. Fresh-frozen plasma remains the mainstay of treatment in the United States, along with adjunct therapies like antifibrinolytic agents.

Other Inherited Factor Deficiencies

Genetic mutations may occur in any of the proteins involved in the formation of the fibrin clot or in regulation of the coagulation pathways. Deficiencies of some of these, such as tissue factor, do not appear to be compatible with life. Mutations of factors other than 8, 9, and 11 are typically very rare, and these are especially heterogeneous with respect to the range of bleeding symptoms (from none to severe).[9]

Deficiencies of factor 12 and other contact factors

Deficiencies of factors in the contact pathway of coagulation are typically autosomal recessive and include F12, prekallikrein (Fletcher factor), and high molecular weight kininogen. These coagulation curiosities produce a markedly prolonged aPTT in the test tube, but cause no bleeding diatheses. This is likely because the contact factors lack a major role in the in vivo clotting process (see article by Kriz and colleagues, elsewhere in this issue). F12 deficiency is the most common cause of an isolated and significantly prolonged aPTT in a nonbleeding patient, after ruling out heparin contamination and lupus anticoagulant (LA). Severe F12 deficiency is rare, and patients may never be identified unless an aPTT is performed for another indication. Although there are few if any clinical consequences, the patient, laboratory, and clinical care team should be aware of the situation so that unnecessary concern is avoided in the future if an aPTT is ordered again by an unknowing caregiver. Deficiencies of the other contact factors are much less common, and confirmation of these deficiencies may be performed by reference coagulation laboratories.

Deficiencies of factors 2 (prothrombin), 5, 7, and 10

In contrast to contact factor deficiencies, decreased activity of factors 2, 5, 7, and 10 often lead to bleeding disorders of varying severity.[10–14] These are all very infrequent, autosomal recessive disorders (incidence of less than 1 in 500,000).

Prothrombin deficiency is not always severe, because there is normally a large molar excess of F2 over the minimum needed to prevent bleeding. Bleeding is seen predominantly in homozygous or compound heterozygous individuals, and can be moderate to severe. In the laboratory, prothrombin deficiency is characterized by both a prolonged PT and aPTT. If clinical suspicion warrants, an F2 activity assay should be performed. Activity testing most often is performed using a one-stage assay. The prothrombin gene is found on chromosome 11p11.2. More than 40 different mutations have been identified in this deficiency. Given the rarity of this disorder, a clear genotype/phenotype correlation is difficult to ascertain, but the lower the levels, the greater the severity of bleeding; symptoms include mucosal bleeding, surgical and trauma related hemorrhage, hemarthroses, and intracranial bleeding. Treatment of bleeding episodes usually is accomplished by use of plasma or prothrombin complex concentrate (PCC), which contains F2, F7, F9, and F10. The exact amount of F2 in these products is unknown. PCC usually is dosed based on the F9 units in each lot. Hence, the dose of PCC will vary from product to product and may lead to supratherapeutic levels of the other transfused factors; this may increase the risk of thrombosis. Thus, PCC dosing is limited to less than 200 U/kg/d. Monitoring of this therapy is done by following the PT/aPTT or by specific F2 assays.

The clinical variability of F5 deficiency is complicated by the fact that F5 is found within platelet alpha-granules and in plasma. Aside from mutations that give rise to inherited F5 deficiency, deficiencies of F5 also can be acquired secondary to F5 inhibitors that result from exposure to bovine thrombin. Activated F5 serves as a cofactor in the prothrombinase complex (like F8 in the tenase complex) that cleaves and activates F2. Like F11, the F5 activity level has limited correlation with the severity of bleeding, but the lower the

level, the greater the severity of bleeding. Patients who are seen clinically for bleeding usually have levels less than 5%. Data from the bleeding registries suggest that these patients usually present with skin and mucosal bleedings, but the more severely affected may present with CNS bleeds, hemarthrosis, or muscular bleeds, which explains why F5 deficiency has been termed parahemophilia. The gene for F5 is located on chromosome 1q23. More than 60 mutations associated with F5 deficiency have been identified. Similar to F2 deficiency, F5 deficiency is characterized by prolongation of both the PT and aPTT. If a low F5 is found, then inherited F5 deficiency must be distinguished from DIC, liver disease, combined deficiencies, or an acquired inhibitor. Plasma is the mainstay of therapy in these patients; however, platelet transfusions also may be beneficial in resistant bleeding cases and for outpatient therapy.

F7 deficiency is the most frequent among the more rare congenital bleeding disorders, characterized by an isolated prolongation of the PT. The gene is located on the long arm of chromosome 13. To date, more than 130 mutations have been reported. Similar to other rare bleeding disorders, bleeding in these patients is heterogeneous both in regards to site and severity, and the correlation between circulating F7 and clinical bleeding is not linear. Patients who present early in life (younger than 6 months) or have CNS or gastrointestinal (GI) hemorrhage or hemarthrosis, however, are clearly considered to be severe cases. The F7-dependent PT clotting assay is easily available, but nuances in the influence of different thromboplastin reagents on the specific assay must be considered. For example, if the PT with a purified thromboplastin reagent is prolonged in an African American patient (especially without a history of clinical bleeding), a F7 polymorphism may be present that actually yields a normal PT result using recombinant human thromboplastin. Several F7 plasma-derived products are available in the European Union to treat severe bleeding cases, but therapy is complicated by the short in vivo half-life of F7, especially in children who are the most severely affected. Hence, these products have to administered frequently. In addition to plasma-derived F7 products, the recombinant form of activated F7 is also available (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark) worldwide.

F10 is a liver-produced serine protease that serves a pivotal role as the first enzyme in the common pathway of clot formation. Patients who have severe deficiency tend to have the most severe symptoms, and patients are classified similar to hemophilia as severe (less than 1% of F10 clotting activity), moderate (1% to 5%), and mild (6% to 10%). Severe F10 deficiency is one of the most rare disorders. The gene for F10 is located on chromosome 13q34-ter. More than 80 different mutations have been identified thus far. In contrast to F8 and F9 deficiency, the most frequent bleeding manifestations are mucocutaneous, including severe menorrhagia. Hemarthrosis also has been reported in rare patients. Patients who have severe deficiency may present in the newborn period with bleeding after circumcision, from the umbilical stump, or with GI or CNS hemorrhage. The diagnosis should be suspected when both the PT and aPTT are prolonged; the F10 functional assay will reveal the deficiency. Like F11, F10 levels are lower at birth, and do not approximate adult values till after 6 months of age. F10 replacement therapy is achieved with plasma or PCC (dosing is again by F9 units).

Deficiencies of factors 1 (fibrinogen) and 13

Fibrinogen (F1) is neither a cofactor nor an enzyme, but a glycoprotein produced in the liver that serves as a substrate to make fibrin clots. Unlike factor assays, which generally are reported as activity, the laboratory typically reports fibrinogen *concentration*, rather than activity. Prothrombin (F2) cleaves two small fibrinopeptides from fibrinogen, allowing it to spontaneously aggregate. In normal physiology, these cleavage products polymerize noncovalently to form a fibrin clot that subsequently is cross-linked covalently by

F13. It is one of these covalent bonds between the D regions of two fibrin molecules that produces the D-dimers measured in the laboratory after degradation of the clot during fibrinolysis. Fibrinogen contains many specific binding sites and plays other adhesive roles in addition to that of a substrate in secondary hemostasis. Fibrinogen interacts with platelet GPIIb-IIIa, fibronectin, and collagen in cell-based hemostasis. Inherited disorders can manifest as quantitative defects (afibrinogenemia/hypofibrinogenemia) or qualitative defects (dysfibrinogenemia), and clinical manifestations vary from asymptomatic to life-threatening bleeds, and paradoxically even to thromboembolic events.

Afibrinogenemia often is diagnosed in the newborn period with umbilical cord bleeding or other severe life- or limb-threatening bleeding. Hypofibrinogenemia presents with lesser bleeding episodes and usually is diagnosed after a challenge to the hemostatic system, while dysfibrinogenemia is diagnosed commonly during adulthood and also may be acquired with liver disease. In afibrinogenemia, all coagulation tests that depend on fibrin as the endpoint (PT, aPTT, thrombin time, reptilase time) are prolonged infinitely, and fibrinogen is undetectable by both functional (von Clauss) and antigenic assays. A fibrinogen level less than 100 mg/dL often will translate to prolonged PT and aPTT. In hypofibrinogenemia, the thrombin time is a very sensitive test, and is confirmed by an abnormal reptilase time. In dysfibrinogenemia, there is usually a discrepancy between clottable protein and antigenically measured fibrinogen. Replacement therapy is generally effective in treating bleeding episodes caused by any of the fibrinogen disorders, including cryoprecipitate and plasma-derived fibrinogen concentrate (RiaSTAP, CSL Behring, King of Prussia, PA, recently approved by the US Food and Drug Administration).

Inherited dysfibrinogenemia is an autosomal-dominant type 2 defect, with several identified mutations. The thrombin and reptilase times are prolonged. The former is assayed after the addition of thrombin to patient plasma, directly converting fibrinogen to fibrin. The thrombin time is sensitive to any deficiency of functional fibrinogen but is also very sensitive to heparin, which greatly increases the inhibition of added thrombin by endogenous antithrombin. The reptilase time is also sensitive to fibrinogen deficiencies. Reptilase is a thrombin-like molecule derived from snake venom that directly cleaves fibrinogen and allows clot formation, but is not affected by heparin, as reptilase is not inhibited by antithrombin.

Deficiency of F13[15] leads to an unstable clot that may be dissolved or dislodged by trauma. The short life of the uncross-linked clot leads to symptoms of delayed bleeding, often presenting as late umbilical cord bleeding (and poor wound healing). A specific F13 activity level is performed by some reference laboratories, but the most common test used to screen for homozygous (severe) F13 deficiency is urea clot lysis. Urea is a chaotropic agent that enhances the dissociation of the uncross-linked fibrin molecules in patients who have severe F13 deficiency. This test involves clotting the plasma, adding 5 mol/L urea, then assessing the time to clot dissolution. The thromboelastograph also can be used to diagnose F13 deficiency as evidenced by reduced maximal amplitude and a rapid decrease in clot size and strength (see article by Teruya in this issue). Plasma F13 is a heterotetramer (FXIII-A2B2). The gene coding for FXIII-A subunit is on chromosome 6p24-25, while that for the FXIII-B subunit is on chromosome 1q31-32.1. Congenital deficiency can be caused by defects in either FXIII-A (type 2 defect), usually resulting in clinical bleeding, or FXIII-B (type 1 defect), where bleeding occurs infrequently. More than 70 subunit gene mutations have been identified (67 in subunit A, only 4 thus far in subunit B). In those homozygous patients with levels that are either absent or less than 5% (variability caused by assay inaccuracy), F13 deficiency is associated with severe bleeding, spontaneous intracranial hemorrhages, poor wound healing, and spontaneous abortions.

Heterozygotes are usually asymptomatic. Replacement therapy includes cryoprecipitate and a plasma-derived FXIII concentrate (Fibrogammin, ZLB Behring, Marburg, Germany) approved in the European Union, but also available on a compassionate basis in the United States at the time of this writing.

Combined factor deficiencies

Many rare combined factor deficiencies have been identified in various pedigrees (see **Table 1** for examples). In the laboratory, if a single factor deficiency is diagnosed but does not explain all of the abnormal screening tests, the presence of an additional deficiency should be considered. Combined deficiency of both F5 and F8 can result from mutations in either the LMAN1 (located on chromosome 18q21) or MCFD2 (located on chromosome 2p21) genes encoding proteins that shuttle these factors from the endoplasmic reticulum to the Golgi complex. The deficiency is characterized by concomitant low levels (usually 5% to 20%) of both F5 and F8, and it is associated with mild-to-moderate bleeding. Treatment requires both F5 and F8 replacement. Combined deficiency of F7 and F10 has been reported with 13q deletions; the two genes are located very close to each other. Combined deficiencies of the vitamin K-dependent proteins 2, 7, 9, 10 can occur when there is an abnormality in the gamma–glutamyl carboxylase gene or the vitamin K epoxide reductase complex.

ACQUIRED FACTOR DEFICIENCIES

In contrast to the rare inherited deficiencies, coagulation factor deficiencies are acquired commonly.[16] These include deficiencies secondary to autoantibody formation leading to neutralization of factor activity or rarely, accelerated clearance from the circulation.[17] Other acquired causes include decreased production (eg, liver disease), impaired synthesis (eg, vitamin K deficiency), and increased destruction (disseminated intravascular coagulation and thrombolytic therapy).

Immune-mediated Acquired Factor Deficiencies

A complex interaction of several variables leads to inhibitor formation in congenital hemophilia, while acquired hemophilia in genetically normal individuals represents a failure of immune tolerance mechanisms.[18] The development of inhibitor antibodies is perhaps the most serious complication of coagulation factor replacement therapy in congenital hemophilia, while acquired hemophilia represents an uncommon disorder in older adults that is generally responsive to current immunosuppressive regimens.

Alloantibody factor inhibitors in hereditary hemophilia

Inhibitors of congenital hemophilia are alloantibodies stimulated by infusion of exogenous factor that contains epitopes that may not be present on the mutated endogenous factor.[3,17,19] These antibodies complicate the treatment of bleeding, because they bind and inactivate infused factor, or occasionally accelerate its clearance, often rendering standard factor replacement therapy ineffective. Approximately 20 to 30% of severe hemophilia A patients develop inhibitors, and this incidence is even higher in children who have large deletions or inversions. Inhibitors are far less common in moderate and mild hemophilia A and in severe hemophilia B (less than 5% in the latter). Inhibitors cause management of these patients to become more difficult and costly. The antibodies not only neutralize the infused factor but also may cross-react with endogenous factor, sometimes resulting in the conversion of moderate hemophilia to a severe phenotype.

Coagulation laboratories supporting hemophilia centers typically quantitate the titer of inhibitor (Bethesda units (BU)/mL) present using a series of F8 assays that have

been modified to show neutralization of human F8 (**Fig. 1**). In patients who have high inhibitor levels (greater than 5 BU/mL) and severe bleeding, therapies to bypass the defect in the coagulation cascade must be used, because the antibody simply neutralizes the large amounts of factor that are infused. Recombinant activated F7 (rVIIa, Novoseven) and activated prothrombin complex concentrates (FEIBA, Baxter Healthcare, Westlake Village, CA, USA) are the most commonly used bypass drugs.[20] These agents directly stimulate F10 activation without involvement of the missing intermediaries (F8 or F9). The coagulation laboratory should be informed if bypass agents have been administered before any coagulation testing, as these may alter the results and clinical interpretation dramatically.

Autoantibodies causing acquired hemophilia

The laboratory diagnosis of acquired hemophilia typically is made when an isolated factor deficiency is identified in a patient with clinically significant bleeding who has

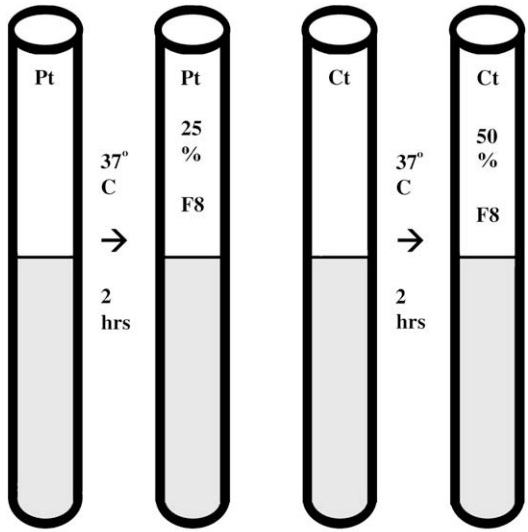

Fig. 1. To quantitate a factor 8 (F8) inhibitor, patient plasma is incubated with a source of F8 (normal pooled plasma) at 37°C for 2 hours. A control (F8 deficient plasma + normal pooled plasma) is also incubated at 37°C for 2 hours. Residual F8 activity is then measured on both mixtures. The residual F8 activity of the patient mixture is compared to that of the control mixture (patient/control). If the resulting ratio is <0.40, the patient sample will need to be diluted in either buffer (Bethesda assay) or F8 deficient plasma (Nijmegen modification). The reciprocal dilution of patient plasma that results in a F8 activity that is 50% of that of the control mixture is defined as one Bethesda unit (BU). The stronger the inhibitor, the greater the dilution required to allow for expression of the F8 activity. In this example, after incubation, the patient's sample had a residual F8 activity of 25% compared to the 50% seen in the control (ratio of patient/control = 0.50). This patient has a 1 BU inhibitor. If the ratio of patient to control is >80%, this suggests no inhibitor (or a clinically insignificant inhibitor).

a negative personal and family history of a bleeding disorder. The most common entities caused by autoantibodies to coagulation factors are acquired hemophilia A,[21,22] followed by prothrombin deficiency complicating the antiphospholipid syndrome, and acquired F5 deficiency after surgical use of bovine thrombin. These coagulation factor deficiencies are uncommon acquired conditions that can lead to serious hemorrhage. Acquired hemophilia A typically presents in older patients who have no prior history of a bleeding disorder; about one-third of these patients will

- have underlying disorders (autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis), have lymphoproliferative disorders such as chronic lymphocytic leukemia,
- be peripartum, or have been treated with drugs such as penicillin and sulfonamides.

Whereas inhibitors that occur with inherited hemophilia A may be treated with heavy immunosuppressive therapy, autoantibodies that develop in patients who have acquired hemophilia A often respond to a single course of therapy (eg, anti-CD20 [rituximab]).[22]

Other acquired inhibitors include prothrombin deficiency, which typically occurs with persistent lupus anticoagulants, and, unlike the underlying procoagulant disorder, F2 deficiency is associated with bleeding that is responsive to plasma or PCC therapy. Rare autoantibodies against F5 or F13 also can cause bleeding.

The development of alloantibodies against bovine F5 that contaminate thrombin preparations used in certain fibrin glues is documented.[11,23] This inhibitor is in addition to antibodies toward the bovine thrombin itself. Antibodies to bovine F2 generally only create in vitro laboratory excitement by significantly prolonging assays using bovine thrombin as a reagent. Conversely, the antibodies against bovine F5 can cross-react with human F5, creating serious acquired bleeding disorders. Recent preparations of fibrin glue and use of recombinant thrombin (eg, Recothrom, ZymoGenetics, Seattle, WA, USA) may lower the risk of developing these antibodies.

Nonimmune-mediated Acquired Factor Deficiencies

Acquired nonimmune causes of factor deficiencies often are detected in the coagulation laboratory. These include the following:

- Increased destruction. Coagulation factors—particularly fibrinogen— may be depleted by rapid factor activation and eventual consumption in conditions such as DIC. Clinical interventions such as artificial heart valves, left ventricular assist devices, and ECMO also may lead to factor depletion in varying degrees. The administration of fibrinolytic drugs in thrombolytic therapy (eg, tissue plasminogen activator) also decreases some factor levels.
- Decreased production. Conditions that lead to decreased production of coagulation factors are seen commonly in the hospital laboratory. A common example of this is liver disease (see article by Ng in this issue).
- Abnormal production. In addition to decreased production, abnormal production of coagulation proteins also is seen in liver disease. Posttranslational modification of fibrinogen, which is detrimental to its function (dysfibrinogenemia), occurs in patients who have liver disease, and also may be seen in the fetus or newborn. Yet, the prime example of abnormal coagulation factor production is iatrogenic and caused by warfarin (Coumadin) therapy. Warfarin removes the ability of the vitamin K-dependent factors (2, 7, 9, 10, and proteins C and S) to bind to the surfaces where coagulation reactions occur. Warfarin acts by interfering with

the vitamin K-dependent gamma carboxylation of these particular factors, resulting in the formation of protein induced by vitamin K absence or antagonists (PIVKA). These modified factors are unable to bind calcium and therefore cannot be anchored to the phospholipid membrane (see article by Ng in this issue). As the proteins cannot bind to the areas of active clotting, the effective concentrations are decreased greatly.

Loss and sequestration. Proteins lost in conditions like nephrotic syndrome include some involved in hemostasis regulation, such as antithrombin. Acquired F10 deficiency is rare; it usually is associated with light chain amyloidosis as a result of accelerated removal of F10 by adsorption onto the amyloid fibrils.[24] An iatrogenic source of factor loss is plasmapheresis, which will lower the coagulation factors by straightforward plasma removal, unless the treatment indication requires plasma replacement (plasma exchange). If plasma is not used as a replacement fluid, fibrinogen (F1) deficiency commonly will limit the frequency of exchanges.

Inactivation. It must not be overlooked that many pharmacologic inhibitors to coagulation factors are entering the clinical arena rapidly. These include the direct thrombin inhibitors (DTI) such as hirudin and argatroban. As might be imagined, inhibition of one of the key factors in the coagulation cascade wreaks havoc upon all of the clot-based assays used in the coagulation laboratory. It is critical that the laboratory be informed when such agents are in therapeutic use to have a reasonable interpretation of coagulation results, including factor assays. A normal thrombin time result is useful in ruling out an effect of DTI.

THE LABORATORY EVALUATION OF FACTOR DEFICIENCY

Preanalytical Considerations

Any coagulation assay is only as good as the sample submitted. Although preanalytical variables can affect any laboratory test, coagulation testing is particularly influenced by collection and handling processes.[25] Inaccurate results caused by improper collection or handling may lead to inappropriate patient intervention and/or additional unnecessary testing. To ensure a quality sample, one must adhere to the current Clinical and Laboratory Standards Institute (CLSI) guidelines for collecting and handling light blue top tubes (H21-A5).[26] **Box 2** outlines a checklist of key preanalytical considerations.

The proper anticoagulant is 3.2% (109 mmol/L) buffered trisodium citrate (light blue top tubes). This blue-top tube is required for most coagulation tests performed by the laboratory. The citrate anticoagulant chelates calcium, thus preventing the sample from clotting. For accurate results, a blood-to-anticoagulant ratio of 9:1 must be maintained. If the patient has a hematocrit of greater than 55% and thus a significantly reduced plasma volume, the amount of citrate must be reduced similarly to prevent falsely prolonged clotting times because of a relative excess of citrate. Too little citrate (as seen when the hematocrit is less than 20%) may not prevent factor activation or clotting in the tube. For this reason, it is recommended that patient samples should be checked for a clot whenever clotting times are inexplicably longer or shorter than either the reference or therapeutic range.

If the sample is collected using a winged blood collection set (ie, butterfly) and a vacuum tube, a discard tube should be drawn first to prevent underfilling of the tube because of the extra air within the collection set. Drawing a discard tube first is also advisable when drawing samples for any coagulation assay beyond routine PT and aPTT testing, although there are no current studies proving this is necessary.

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Box 2
Preanalytical checklist. These questions should be asked as part of the preanalytical checklist to determine sample integrity

- Was the specimen collected in the correct anticoagulant?
- Are the tubes properly filled?
- Was the sample collected through a venous access device?
- Is the hematocrit less than 55%?
- Is the sample already clotted?
- Is the plasma platelet-poor?
- Is the sample hemolyzed?
- Is the sample lipemic or icteric?
- Has the sample been maintained either at room temperature or frozen?
- Has the sample age exceeded the stability limit?

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Within 4 hours of collection, (1 hour for assays evaluating heparin), the blood must be centrifuged and plasma processed for testing or freezing. A two-spin centrifugation method will ensure that the resulting plasma is truly platelet-poor, because phospholipid from residual platelets interferes with many coagulation assays. Plasma should be kept at room temperature until testing or freezing, because both vWF and F8 activity can be lost with 4°C storage. Frozen plasma must be stored at -20°C or lower (preferably -70°C), and avoiding the use of a frost-free freezer, as the periodic defrost cycles allow samples to partially thaw and then refreeze. Frozen plasma should be thawed at 37°C just before testing, avoiding any refreezing of plasma, as the coagulation proteins tend to denature with more than one freeze–thaw cycle.

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Using the Prothrombin Time and Activated Partial Thromboplastin Time to Screen for Factor Deficiency

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Establishing reference ranges

To successfully use the PT and aPTT as screening tests, the coagulation technologist needs to be very familiar with the characteristics of the reagent/instrument systems. Selecting a combination of coagulation reagents and analyzers that facilitate the identification of patients with factor deficiencies or inhibitors is critical. The aPTT reagents touting lupus anticoagulant sensitivity should be reserved for actual LA testing. For PT testing, it is preferable to use a low International Sensitivity Index (ISI) reagent containing a heparin neutralizer; the latter enables accurate PT results in patients who are receiving both warfarin and heparin. The neutralizer generally is effective when heparin levels are less than or equal to 1 U/mL, but can be overwhelmed by contaminant heparin when specimens are drawn from an indwelling line.

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To establish a valid reference range for the PT and aPTT, a pool of normal subjects must be identified. These subjects should match the patient population as closely as possible. This may be difficult if the population includes pediatric patients. In the absence of a neonatal or pediatric pool of donors, one must rely on published reference ranges for children of various ages.[6,27] Adult outpatients can be used to establish ranges as long as they do not have orders for coagulation assays (suspected bleeding or clotting history) and additionally meet predefined criteria (**Box 3**). Historically, donors who were on oral contraceptives (OCPs) were excluded from the normal donor pool, but many sites now include them, because they constitute a large portion of the population to be tested,

Box 3
List of donor requirements for use as normal donor plasma. Criteria for inclusion in a normal reference range study

- Not pregnant
- Not taking anticoagulants
- Not taking antibiotics
- Not taking insulin
- No history of a blood clot
- No history of a bleeding disorder
- No history of autoimmune disease (eg, lupus erythematosus, rheumatoid arthritis)

and newer OCPs contain less estrogen. Estrogen may increase ambient F8 and vWF levels, thus shortening the aPTT in affected patients. This is illustrated from data collected from students at the authors' institution, where the average aPTT of women using OCP was 27.6 seconds, n=18, while for women not on OCP, the average aPTT was 29.5 seconds, n=14, (*P*=.026). For men the average was 29.4 seconds, n=18.

Ideally, donor samples should be collected and tested over a period of days or weeks. They should be centrifuged and analyzed in the same manner as patient samples. A bare minimum of 20 donors (10 male and 10 female) can be used to verify the reference range of a new lot of a current reagent; however, when changing type of reagent, at least 50 donors should be used. Statistical analysis also will determine the required geometric mean PT value for international normalized ratio (INR) calculation.

In order to interpret results of the PT and aPTT, it is helpful to know their sensitivity for factor deficiencies. To evaluate a reagent for factor sensitivity, one may dilute a pooled normal plasma into factor-deficient plasma to achieve factor activities of 60%, 50%, 40%, 30%, and 20%. The appropriate factor assay then is performed, as well as a standard PT and aPTT on each dilution (**Fig. 2**). The percent activity is plotted against the clotting time in seconds to determine what clotting time corresponds to a factor activity level of 30%, which generally is considered to be the minimum amount of factor activity needed for consistent hemostasis (**Table 2**).

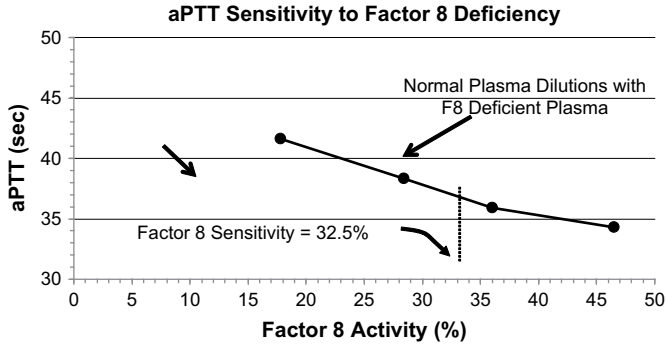

Fig. 2. Determining the upper limit of the activated partial thromboplastin time (aPTT) reference range is aided by measuring the factor 8 activity against the aPTT. Using dilutions of control plasma, a factor 8 activity of approximately 32.5% confirms the statistically-derived upper limit of normal of 37.0 seconds.

Table 2
Half-lives and hemostatic levels of coagulation factors

Factor	Category	Approximate Half-Life	Approximate Hemostatic Level
Fibrinogen	Substrate	96 hours	50 mg/dL
F2	Protease	72 hours	20%
F5	Cofactor	16 hours	25%
F7	Protease	4 hours	20%
F8	Cofactor	11 hours	30%
F9	Protease	22 hours	30%
F10	Protease	30 hours	25%
F11	Protease	60 hours	25%
F13	Transglutaminase	10 days	2% to 3%

Some table content courtesy of George Fritsma (www.fritsmafactor.com).

The reference ranges for both PT and aPTT should be established for each lot of reagent by examining both the statistical analyses and the results of the factor sensitivity studies. The upper and lower limits of normal should initially be set at the 95th percentile (mean plus or minus 2 SD). The upper limit can then be adjusted so that true factor deficiencies (less than or equal to 30%) will be detected. If possible, patient plasmas known to be mildly factor-deficient should be assayed to confirm that the reference ranges have been set correctly. This procedure is also useful in circumstances where the in vitro factor sensitivity curve looks relatively flat in the 30% range (eg, the F9 curve is relatively insensitive in some reagent systems). Plasma from patients with mild factor deficiencies should be frozen for future use in setting reference ranges. The factor sensitivity information should be supplied to hematologists, pathologists, and all other providers who routinely use the PT or aPTT for screening.

Mixing studies

To guide in the evaluation of a prolonged PT or aPTT, mixing studies can be employed to help discern the presence of factor inhibitors. In its simplest form, one part of patient plasma is mixed with one part of pooled normal plasma, and the clotting tests are repeated. Pooled normal plasma supplies the missing factor(s) and corrects the prolonged clotting times of patients who have factor deficiency. If a patient has an inhibitor (antibody) that interferes with factor activity, the prolonged clotting time should not correct with addition of normal pooled plasma. A 1- to 2-hour incubation at 37°C is needed for time- or temperature-dependent antibodies to exert their effect. A normal control must be used for interpretation, as extended incubation may prolong the clotting time of plasma because of factor degradation, without regard to inhibitors or deficiencies.

Exactly defining a mixing study correction can be difficult, and there are many opinions as to how this should be accomplished.[28,29] Some experts suggest that the result of the mix must correct to within the reference range, while others hold that correction occurs if the mix result is no greater than 5 seconds above the upper limit of the range. Others compare the results of the mix with a control of normal pooled plasma. Using various dilution ratios of patient plasma to pooled normal plasma or incorporating saline into the mix may increase the sensitivity of the study for an inhibitor.

In practice, in the authors' laboratories, mixing studies are often misleading.[28] Severe factor deficiencies may not correct even though factor levels have been

restored to 50%, depending on where the reference ranges are set in relation to factor sensitivities. This is especially true with current PT reagents, which are very sensitive; restoring a factor level to 50% may yield a PT within the reference range. Mixing also may dilute a mild inhibitor sufficiently, such that clotting times correct. With a good clinical history and knowledge of one's laboratory test limits, an experienced coagulation laboratory may be able to determine the cause of a prolonged PT or aPTT without mixing studies.

Factor activity assays

Three types of assays can be used to measure factor activity: chromogenic, one-stage clotting, and two-stage clotting. Although expensive, chromogenic assays are superior for measuring factor activity in the presence of LA or heparin, in assigning factor concentrate potency, and for detecting mild F8 deficiencies that may be missed with a one-stage clotting assay.

Chromogenic assays are based upon the principle that clotting factors are proteinases that specifically cleave their natural substrates. By substituting a chromogenic substrate that gives off a color when cleaved, the reaction rate and the magnitude of color development are proportional to the factor activity. Each chromogenic assay is designed specifically for the factor being measured. For example, one method for F8 uses optimal amounts of Ca^{2+}, phospholipid, and activated F9, with an excess of F10, such that the rate of activation of F10 is related linearly to the amount of F8. F10 hydrolyzes the chromogenic substrate S-2765, thus liberating a chromophore. The color then is read photometrically at 405 nm. The intensity of color is proportional to the amount of S-2765 cleaved by F10 and thus yields the F8 activity in the sample.

Two-stage clotting assays are precise, insensitive to preanalytic factor activation, and superior at detecting mild F8 deficiency compared with the one-stage assay. Two-stage assays do not require the use of factor-deficient plasmas, but they are complicated and not automated easily. For this reason, they are not performed in many laboratories. In the two-stage method for F8, the patient plasma is treated with aluminum hydroxide to remove factors 2, 7, 9 and 10. This arrests the clotting process after formation of the prothrombinase complex. The treated patient's plasma then is mixed with activated serum, F5, calcium, and phospholipids to initiate coagulation and generate activated F10. After a defined period of incubation, one volume of this mixture is added to one volume of normal plasma, and the time to clot formation is measured. Clotting times of a calibrator or standard plasma are plotted, and the F8 level in the patient sample is read from the graph.

The one-stage clotting assay is the most commonly used method in clinical laboratories because of its simplicity and automation. Factors 8, 9, 11, and 12 typically are tested using a one-stage assay based on the aPTT. F7 is tested using an assay based on the PT, while factors 2, 5, and 10 can be assayed using either. The PT-based assay is preferred, however, because it is faster and less prone to interference by heparin and LA.

Regardless of whether a PT- or aPTT-based assay is used, a calibration curve first must be prepared. It is important to choose a good calibrator referenced to World Health Organization standards. The standard curve should be designed to contain enough points to extend below 1% (especially for factors 8, 9, and 11) so that severe hemophilia can be differentiated from moderate disease. Some automated coagulation analyzers require the use of a low curve to enhance sensitivity below 20% activity. The low curve is made by diluting the standard down to approximately 20% before loading it on the analyzer. The analyzer then can prepare dilutions to include a point at 1%.

To make factor assays more sensitive, patient and calibrator plasma first are diluted in buffer (longer times are more sensitive to small changes); then the diluted plasma is mixed 1:1 with factor deficient plasma. An aPTT or PT then is performed on the mix. Most automated coagulation analyzers prepare all patient and calibrator dilutions without technologist intervention.

Factor-deficient plasma is provided as a lyophilized powder or frozen plasma. Deficient plasma may originate from a single donor who is congenitally factor-deficient, or it may be prepared from pooled normal donor plasma by immunodepletion of the appropriate factor. If an immunodepleted plasma is used, it should be validated to ensure that only the factor under consideration has been removed. With F8-deficient plasma, it is important to know if vWF also has been removed, as the absence of vWF may affect the results of the factor activity assay.

To rule out interference from heparin, LA, or antibodies to another factor, each sample must be tested at a minimum of three dilutions. If the results, after correcting for the various dilutions, exhibit increasing activity with each subsequent greater dilution of the patient plasma, this is termed an inhibitor pattern (**Fig. 3**). It is important to note that an inhibitor pattern may not be observed when an antibody is directed specifically against the factor being tested. Specific factor inhibitors usually bind to the factor and completely interfere with its coagulation function, preventing it from being measured in an activity assay. Subsequent dilutions do not produce a rise in factor activity.

Heparin causes aPTT-based assays to have prolonged clotting times (and thus falsely low factor activity). When the patient plasma is diluted out, the heparin also is diluted, causing less interference in the assay and resulting in an apparent increase in factor activity. A prolonged thrombin time with a normal reptilase time (or measuring the actual heparin level) will confirm heparin as the cause of an inhibitor pattern.

LA may show an inhibitor pattern in a manner similar to heparin. The antibody interferes with phospholipid in the PT- or, most often, acPTT-based assays and leads to prolonged clotting times. As the patient plasma is diluted, the antibody also is diluted, causing less interference and thus higher—and more accurate—factor activity with

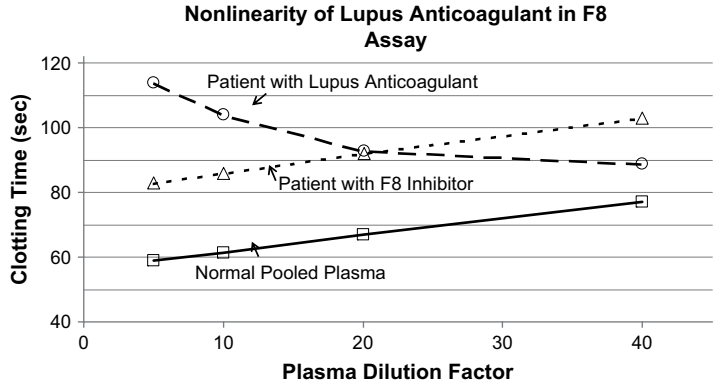

Fig. 3. Lupus anticoagulant (LA) pattern with serial plasma dilutions. The normal pooled plasma shows a straight-line pattern; by contrast, the lupus anticoagulant inhibitor line is nonlinear, with the clotting time shortening at a greater pace with increasing dilutions. This pattern suggests that the inhibitor effect is being diluted out. Note that the a patient with a factor 8 inhibitor generally runs parallel to the normal pooled control, though at an increased clotting time due to the low amount of factor 8.

each dilution. A positive dilute Russell viper venom test (DRVVT) will confirm the diagnosis of LA, but a negative DRVVT does not rule out its presence. A hexagonal phase phospholipid neutralization (eg, StaClot LA, Diagnostica Stago, Asnieres Sur Seine, France), platelet neutralization procedure, or other assays for LA may be needed to confirm the presence of a LA inhibitor.[30]

If a patient has an antibody to a factor other than the one being assayed, it may react with that factor which is present in the factor-deficient plasma and thereby causing prolongation of the PT- or aPTT-based factor assay. As the patient's plasma is diluted, the antibody also is diluted, resulting in less interference and a gain in factor activity with each subsequent dilution. This produces a nonlinear curve compared with the calibration curve.

Assays for detecting specific factor inhibitors

Bethesda-type assays (or its modification, the Nijmegan assay) can be employed to screen for inhibitors to factors.[31] Although predominantly used for F8 inhibitors, these assays can be adapted to detect any factor inhibitor. In a Bethesda assay, patient plasma is incubated with a source of factor (usually normal pooled plasma) for 2 hours at 37°C. Residual factor activity then is measured and compared with a control mixture. One Bethesda unit is defined as the amount of antibody that will inhibit 50% of the available factor in the normal pooled plasma. It may be necessary to dilute the patient plasma in buffer or factor-deficient plasma before incubation to achieve a mixture with the optimal range of 40% to 60% residual factor activity. The residual factor activity is converted to BUs by the use of a Bethesda graph and then multiplied by the dilution factor (see **Fig. 1**).[31]

The control mixture should mimic the patient mixture as closely as possible. If the patient has a low titer inhibitor (less than or equal to 1 BU), the control should be composed of normal pooled plasma and factor-deficient plasma. If the patient has to be diluted in buffer, the control should be made using normal pooled plasma and buffer. In the Nijmegan modification, the use of buffered normal pooled plasma stabilizes the pH, permitting more accurate measurement of low-titer inhibitors. Using the lowest possible patient dilution that gives a 40% to 60% residual factor activity prevents overestimation of inhibitors possessing complex kinetics.

The inhibitor testing protocol is accepted widely for use in patients who have no circulating factor activity (less than 1%). When the patient has measurable factor activity in the sample, however, there is less agreement on protocols. Although there is some evidence that heat-treating the plasma before incubation with normal pooled plasma will inactivate the patient's own factor, but not the antibody, the World Federation of Hemophilia Laboratory Manual recommends either adjusting the concentration of the control solution to match that of the patient or mathematically correcting for the baseline factor activity of the patient.[32]

Laboratory Evaluation of a Prolonged Prothrombin Time or Activated Partial Thromboplastin Time

Having access to the patient's clinical, medication, and transfusion history is one of the best coagulation screening tests, and often may eliminate the need for mixing studies to guide test selection.[33] Is the patient actively bleeding or bruising? Is the patient anemic? Is there any personal or family history of bleeding? If the answer to any of these questions is yes, it makes sense to look for factor deficiencies (either congenital or acquired) and vWD.

If there is a history consistent with clotting (eg, deep vein thrombosis, pulmonary embolism, stroke, or spontaneous abortion), or if the prolonged screening test is the only reason for workup in an asymptomatic patient, this favors LA testing.

Medication history will reveal if the patient is receiving warfarin or heparin or another anticoagulant such as a direct thrombin inhibitor. Does the patient have a venous access catheter? If so, screen for heparin or consider a dilution effect. If there is an isolated prolonged PT, one must ask: Has the patient been given vitamin K, been on antibiotic therapy, or taken any herbal or natural therapeutics? Although most clinicians expect a prolonged PT/INR in vitamin K depletion, an accompanying prolongation of the aPTT may surprise them and trigger a workup for prolonged aPTT. Most aPTT reagents are fairly sensitive to the low levels of F9 seen in vitamin K depletion, and the decreases in factors 2 and 10. Thus, it is not unusual for the aPTT to be somewhat prolonged with warfarin or other causes of vitamin K deficiency.

One always must investigate the patient's transfusion history. Has the patient received any plasma, cryoprecipitate, bypassing agents, or factor concentrates? If so, coagulation studies may be difficult to interpret. Bypassing agents contain activated F7, which can cause extremely short clotting times (and thus overestimation of factor activity levels).

Patients may have more than one coagulopathy. For instance, combined F5/F8 deficiency is a well-characterized familial defect. These patients will present with a prolonged PT and aPTT and usually will have bleeding symptoms when challenged.

Isolated prothrombin time elevation

Once heparin, warfarin, and other anticoagulants are ruled out, an isolated elevation of the PT/INR likely has one of several etiologies (**Table 3**):

Vitamin K deficiency. The most frequent cause of an elevated PT is vitamin K deficiency. Some herbal or natural products also may induce vitamin K deficiency. Patients on antibiotics are susceptible to vitamin K deficiency, because the antibiotics may destroy gut bacteria that synthesize vitamin K. Because F5 is not

Table 3
Isolated prothrombin time elevation (assumes that heparin, warfarin, and other anticoagulants have been ruled out)

Differential Diagnosis	Tests to Run	Interpretation/Follow-up
Vitamin K deficiency Liver disease F7 deficiency (or inhibitor), Lupus anticoagulant	Factors 5, 7, and LA panel Recommend repeat draw to verify low factor levels	If both F5 and F7 are ↓, suspect liver involvement If only F7 is ↓, run at least one more vitamin K-dependent factor If ↓, suspect vitamin K deficiency If only F7 is ↓, suspect congenital deficiency or acquired inhibitor (rare) Consider Bethesda assay if no family history and recent onset of bleeding If lupus anticoagulant positive, repeat in 3 months to confirm persistence and therefore significance

vitamin K- dependent, assaying F5 along with one or several of the vitamin K dependent factors will help differentiate vitamin K deficiency from liver disease. It is helpful to be familiar with the half-lives of the various factors when interpreting results of coagulation assays (see **Table 2**). As a rule, the shorter the half-life, the faster the decrease in activity when starting warfarin, and the faster the recovery of activity when discontinuing warfarin. In early vitamin K deficiency, only the F7 may have fallen enough to prolong the PT. If inflammation is present, acute-phase elevations of F8 and fibrinogen often keep the aPTT from prolonging. Additionally, when more than one factor is decreased, the PT may prolong more than the single factor sensitivity studies would predict.

Factor 7 deficiency. The degree of correlation between the F7 activity level and patient bleeding varies depending on the type of tissue factor used in the F7 assay. An inhibitor to F7 is rare and is usually attributable to IgG autoantibodies. A Bethesda-type assay can be performed to confirm the presence of an F7 inhibitor.

Lupus anticoagulant. Although more commonly associated with an elevation of the aPTT, LA may produce an isolated elevated PT. The source of phospholipid and phospholipid content of the PT reagent will define how sensitive the reagent is to LA. Elevated F8 or fibrinogen may keep the aPTT from prolonging. One should consider LA testing on any sample with unexplained prolongation of the PT.

Isolated activated partial thromboplastin time elevation

After excluding LA, the clinical picture as well as the personal, family, and medication history (including anticoagulant therapy) will guide the workup of an isolated prolonged aPTT (**Table 4**).[33] With a history of bleeding, assays for F8 activity, vWD, F9, and F11 are most informative. If any factor activity level is low, one should evaluate at least three patient dilutions for an inhibitor pattern. If any one factor shows very low activity that does not increase with patient dilution, a Bethesda-type assay is warranted. Patients also can have more than one disorder responsible for the prolonged aPTT (eg, F8 deficiency or F8 inhibitor with a LA). The presence of LA also can make it challenging to monitor replacement therapy with clot-based assays. A chromogenic F8 assay may give a more accurate factor result in the presence of LA.

Table 4
Isolated elevated activated partial thromboplastin time (aPTT) (assumes heparin and other anticoagulants have been ruled out)

Differential Diagnosis	Initial Tests to Run	Interpretation/Follow-Up
Von Willebrand disease (VWD) Factor deficiency Factor inhibitor	If positive history for bleeding or bruising do F8, then F9, then F11	If only F8 slightly ↓to moderately ↓, then run von Willebrand factor antigen and activity If not vWD, do F8 inhibitor If F8, F9, or F11 is markedly ↓, do Bethesda assay for that factor
Lupus anticoagulant (LA)	If negative history for bleeding or bruising and • moderately ↑ aPTT, then do LA, then, F8, F9, F11 • markedly ↑↑ aPTT, then do F12, then consider other contact factors	If positive LA and factor activity does not normalize with additional dilutions, may need to do chromogenic assay If positive LA, repeat in 3 months to confirm persistence and therefore significance

If there is no history of bleeding, and LA is not present, assays for F12 and other contact factors may explain the elevated aPTT.

Combined elevated prothrombin time and activated partial thromboplastin time

Congenital deficiencies or acquired inhibitors of factors 2, 5, or 10 are rare. Laboratory workup should begin with testing the common pathway factor activities, as well as LA testing (**Table 5**). It is not uncommon for patients who have LA to produce antibodies to prothrombin, and approximately 30% of these will result in a prothrombin deficiency with some degree of bleeding. A laboratory phenomenon coined lupus cofactor effect frequently occurs in patients who have hypothrombinemia–lupus anticoagulant syndrome. When patient plasma is mixed with normal pooled plasma, the clotting time (PT, aPTT, DRVVT, or LA-PTT) actually prolongs to an even greater extent. When this phenomenon is noticed in LA testing, a F2 activity assay should be performed.

Decreased F5 activity is a good predictor of liver disease and is used commonly in evaluating liver toxicity in acetaminophen overdose. Because F8 and fibrinogen are acute phase reactants, they often are increased in liver disease, which may keep the aPTT from prolonging even with decreased synthesis of F2, F5, F9, and F10.

Each laboratory's approach to diagnosing factor deficiencies and inhibitors must be flexible. Not all patients have well-defined coagulopathies,[33] and many, especially hospitalized patients, have more than one issue at a time. For example, patients who have congenital or acquired hemophilia have been known to develop LA. Workups must be conducted as efficiently and timely as possible to be of help to the patient and clinician. Abnormal results should be verified on fresh samples (new draws if possible), as there is ample opportunity for preanalytical error.

The laboratory approach to inherited and acquired coagulation factor deficiencies requires active use of clinical information. The coagulation pathologist and hematologist should be familiar with the methods and capabilities of their laboratories. An understanding of the laboratory approach to evaluating factor deficiency not only

Table 5
Combined elevated prothrombin time and activated partial thromboplastin time (assumes heparin and other anticoagulants have been ruled out)

Differential Diagnosis	Initial Tests to Run	Interpretation/Follow-Up
Vitamin K deficiency	F2, F5, and LA	If only F2 ↓, with + LA, look for lupus cofactor effect
Liver disease		If LA-negative, rule out vitamin K deficiency
Disseminated intravascular coagulation (DIC)		Do F7 or 10 and if normal test for F2 inhibitor
Lupus anticoagulant (LA) with hypoprothrombinemia		If only F5 ↓, do F5 inhibitor and do F8
Congenital factor deficiency	F10	Rule out DIC
Factor inhibitor		If only F10↓, look for evidence of amyloidosis, respiratory infection, and malignancy
		Do F10 inhibitor
		If F7 and F2 are ↓ but F5 is normal, likely vitamin K deficiency/warfarin therapy
		If multiple factors are ↓, look for liver disease or DIC

will aid clinicians in obtaining a prompt diagnosis, but also avoid pitfalls for false diagnoses in coagulation testing.

ACKNOWLEDGEMENT

The authors wish to acknowledge the timely contributions of the Special Coagulation technologists at TriCore Reference Laboratories in preparing this manuscript: Pam Owens, Donna Rospopo, and Trish Lowery.

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