Hemophilia diagnosis: how to test, what to know

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March 2017—True, hemophilia is no longer commonly known as the "royal disease" (as it was when several generations of European rulers suffered from it). But in a January webinar, Dorothy M. Adcock, MD, gave some royally important suggestions regarding the laboratory diagnosis of hemophilia A and B.

"In the evaluation of non-severe hemophilia A, it's important to evaluate both the one-stage clot-based factor activity and the chromogenic [assays]," said Dr. Adcock, who is medical director of Colorado Coagulation, of Englewood, Colo., a member of the LabCorp Specialty Testing Group. "Results should always be confirmed on a new plasma sample, and then if present, you should consider molecular testing to identify the underlying mutation." Recommendations on the initial evaluation of non-severe hemophilia B are forthcoming, she added; in the meantime, "please do not rely on an abnormal PTT to screen for non-severe hemophilia A or B."

Hosted by CAP TODAY and sponsored by Novo Nordisk, the webinar—"Laboratory Diagnosis and Therapeutic Monitoring in Hemophilia: Problems, Pitfalls, and Testing Pearls"—saw Dr. Adcock and others discussing issues, challenges, and solutions related to the laboratory assessment of hemophilia A and B. (The webinar is at www.captodayonline.com and additional coverage will be published.)

As Dr. Adcock reminded the audience, there are three methods for measuring the factor deficiencies that define hemophilia A (factor VIII) and B (factor IX): the one-stage clot assay, which is based on activated partial thromboplastin time; the two-stage clot assay, which is rarely performed since it is complex, cannot be automated, and no kit for it is available; and the chromogenic substrate assay, which has limited availability and is often performed as a batched analysis.

Most clinical laboratories use the one-stage method for all factor activity assays. Though the assay is largely standardized, "the many instrument reagent combinations available lead to variability," Dr. Adcock noted. Chromogenic factor VIII and factor IX activity assays are available. Though the former are available in FDA-approved kits from multiple vendors, few laboratories offer the tests. And few labs offer factor IX activity assays by the chromogenic method, which are not available as FDA-approved kits.

"Well, you're probably wondering, does assay methodology used to measure factor activity matter?" Dr. Adcock said. "In fact, it does."

That's been known since the late 1980s, when discrepant non-severe hemophilia A was recognized and described as a greater than twofold difference in results between the one-stage and chromogenic factor VIII activity assays. As many published studies have confirmed, "in discrepant hemophilia A, the one-stage assay result may be greater than the chromogenic assay or the chromogenic result greater than the one-stage assay, and this may impact both diagnosis as well as classification of disease severity," she added.

Discrepant hemophilia has been reported to occur in up to 30 percent of mild or moderate hemophilia A, but has only recently been described in abstract form in a very small cohort of hemophilia B patients. Discrepancies in activity based on assay methodology are also reported in the presence of some new recombinant factor VIII and IX replacement products.

"The next important question is: Are these discrepancies in results real?" she said. For non-severe hemophilia A, at least, the one-stage and chromogenic discrepancy has been reported to be consistent between family members and consistent in all individuals bearing the same mutation. "Therefore, this discrepancy has a molecular genetic basis," she said, with the variability in results depending on the underlying genetic defect. In post-infusion replacement therapy, the discrepancy depends on the modification of the recombinant factor and its effect on the assays.

Dr. Adcock then reviewed the difference in methods, using factor VIII activity as an example ("factor IX assays are very, very similar," she said). As she noted, in the one-stage factor VIII activity assay, test plasma is mixed with factor VIII deficient plasma. That mixture is combined with the aPTT reagent, which contains phospholipid and a surface or contact activator. To initiate clotting, calcium is added, with the time to clot measured in seconds.

The chromogenic factor activity assay is performed in two stages. First, activated factor X is generated; the amount that's generated depends on the amount of functional factor VIII in the test plasma. "The reagent components and the incubation times vary a little by manufacturer," she said. The first stage is incubated for between two and 10 minutes. Second, the amount of activated factor X generated is determined by its ability to hydrolyze a specific chromogenic substrate viewing a colored substance.

"So the factor activity for each assay is then determined off of a standard curve, and this is referenced against an international standard that has a known factor VIII concentration," Dr. Adcock said. "For the one-stage assay, the result is based on seconds, and for the chromogenic assay, it's based on optical density."

What are the critical differences between these assay methods? In the one-stage assay, the reaction proceeds quickly once calcium is added, and the activated form of factor VIII is present for only a very short period. The factors are present at physiologic concentrations. In contrast, in the chromogenic assay the first stage is incubated for a period of time, and activated factor VIII is generated throughout that incubation period. In addition, the factors are often present in quantities greater than are required to optimize the reaction.

"How does this variation in assay methodology play a role in discrepant non-severe hemophilia A?" Dr. Adcock said. "There are mutations, and these are often missense mutations, which tend to be novel. In those circumstances where the one-stage result is greater than the chromogenic, the mutations tend to be localized to the A1-A2-A3 domain interfaces [of the FVIII molecule]. Mutations in these regions tend to cause activated factor VIII to be unstable, and this causes it to lose its activity. This results in less activated factor VIII that is ultimately generated. These mutations are better detected in the chromogenic assay, where activated factor VIII is generated over a period of time in minutes."

When the one-stage assay result is lower than the chromogenic, she continued, mutations tend to be localized to thrombin cleavage sites or factor IX binding sites. These mutations are thought to be more apparent in the one-stage assay, where the factors are present at physiologic concentrations. "It is also believed that the prolonged incubation time and the excess factor present in the chromogenic assay may, at least partially, overcome these binding defects."

Most cases of discrepant non-severe hemophilia A have high, often normal, factor VIII antigen levels, and these therefore represent dysfunctional proteins. "I suspect that measuring factor VIII antigen levels may provide assistance in the identification of these cases of discrepant hemophilia," Dr. Adcock said.

Again, this discrepancy in results between methods may lead to missed diagnosis or misclassification. "Eleven percent of those are reported to have normal factor VIII activity results with the one-stage assay," she said. "You may also wonder which result is correct. It is generally believed that the lower result correlates better with bleeding tendency and the results of thrombin generation assays, although more study is needed in this area.

"Such discrepancies have recently been described in hemophilia B in a small cohort of patients in abstract form," she continued. "Also, deviations in one-stage results may be seen in some hemophilia patients, depending on the PTT reagent used. There is limited information about hemophilia B, however, to date."

As she emphasized to the audience, it's important not to rely on a normal or an abnormal aPTT to screen for hemophilia: "Depending on the aPTT reagent, the one-stage factor VIII activity may have to fall below 25 percent, for example, and the IX below 15 percent before the PTT prolongs, and this is referred to as reagent responsiveness." This is defined as the level of factor activity that must occur before the PTT prolongs. "So this is just a reminder that a normal aPTT does not rule out mild deficiency of factor VIII, IX, or XI."
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