

Laboratory work-up of acquired hemophilia A

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Summary

The paper presents the major aspects of laboratory diagnosis of acquired hemophilia A (AHA). The author discusses the difficulties encountered in the process of detecting this coagulopathy. The principles of laboratory diagnosis are described and interpretation of the laboratory outcome is presented. The current algorithm of the AHA diagnostic procedure is also suggested.

Key words: acquired hemophilia A, AHA, laboratory diagnostics, acquired hemostasis disorders, hemostasis

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Introduction

Hemophilia A (HA) is either a congenital or acquired bleeding disorder which manifests as bleeding/hemorrhage. The excessive bleeding tendency in HA is the result of the deficiency or complete absence of factor VIII in plasma. Unlike the well-known, genetically determined HA, acquired hemophilia A (AHA) is an autoimmune disease caused by auto-antibodies that neutralize coagulant activity of FVIII. Unlike congenital hemophilia A that affects men (mutation in the *F8* gene on the X chromosome), AHA is a bleeding disorder which manifests in both men and women.

AHA is caused by anti-FVIII neutralizing antibodies (called “inhibitor”) which impair coagulant activity of FVIII. The autoantibodies produced in AHA are mostly IgG class and bind to FVIII epitopes located on its C2 and A2 domains. The mechanism of their anticoagulant action relies on impairment of FVIII interaction with phospholipids (anti-C2 antibodies), disturbances in the formation of the intrinsic tenase complex (anti-A2 antibodies) and most likely also on blocking the binding of FVIII to von Willebrand factor. The kinetics of FVIII interaction with autoantibodies in AHA is

different from the kinetics of FVIII interaction with alloantibodies in congenital hemophilia A with inhibitors. While in congenital hemophilia A, alloantibodies completely abolish the FVIII activity in plasma, in acquired hemophilia A, even with a very high titer of autoantibodies, some residual plasma FVIII activity is detected [1].

The main problem related to AHA management is the delay in diagnosis of the disorder and prompt implementation of therapy. The delay may be caused i.a. by insufficient knowledge of the medical staff (laboratory personnel included) regarding the principles of AHA diagnosis. Patients usually report in emergency to centers/doctors who are no specialists in recognition of coagulation disorders. According to the European Acquired Hemophilia Registry (EACH2) a ≥ 1 week delay in AHA diagnosis affects 34% of patients [2].

Moreover, AHA is a rare disease, with the annual incidence rate estimated at approximately 1.48/1 million, although current data indicates that it may be close to 5–6 per million [3]. The incidence of AHA increases with age, amounting to approximately 0.045/million among children under 16 and 14.7/million in people over 85 years of age [4]. The median age at the time of diagnosis of AHA

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is therefore almost 74 years and the diagnostic procedure is often complicated by the age-related higher incidence rate of coexisting morbidities and medication therapies. The incidence of AHA is markedly higher in two population groups: 1) young women in the age range of 20–40 years (this is closely related to pregnancy, especially to the first 12 months after delivery) and 2) older age groups where AHA is slightly more often detected in men. Approximately half of patients with detectable autoantibodies against FVIII do not have underlying disorders (idiopathic AHA). In approximately 40% of cases, AHA is associated with autoimmune disease, solid malignant tumors, hematologic tumors, allergic diseases or with a drug exposure.

In most cases, AHA manifests as a sudden severe bleeding (in persons who reported no bleeding symptoms previously) which can lead to death within several weeks [3]. Typical for acquired hemophilia A are large subcutaneous hematomas, mucosal bleeding (from the gastrointestinal and genitourinary tract), as well as bleeding from the wounds after surgical procedures and after tooth extraction. Intracranial bleeding may occur but spontaneous bleeding into the joints is very rare in AHA.

As mentioned above, most patients report with severe bleeding and require immediate hemostatic treatment. The best way to stop bleeding in AHA is to administer the so-called by-passing agents (BPA), i.e. recombinant activated factor VII (rFVIIa) or activated prothrombin complex concentrate (aPCC) or recombinant porcine FVIII concentrate. Prompt and correct diagnosis of the bleeding disorder is therefore of utmost importance.

There is an additional challenge to laboratory diagnosticians as regards AHA diagnosis. Most elderly people are burdened with a wide spectrum of comorbidities and are exposed to medication (e.g. anticoagulants) which affects their coagulation system and may significantly interfere with the laboratory picture of AHA. Such cases require an individual diagnostic approach and appropriate testing.

Principles of laboratory diagnosis of AHA

The diagnostic tests for AHA are performed on samples of peripheral venous blood (10–15 ml; collected in tubes with Sodium Citrate, Na₃C₆H₅O₇ x 2H₂O; in a 9:1 ratio) used for routine coagulation tests. Measurements of clotting time, coagulation activity and inhibitor titer are performed in citrated platelet-poor plasma, obtained after centrifugation

of citrated blood samples at room temperature for 10–15 min/1500 g [5, 6]. Importantly, tests should be carried out in blood samples taken before initiation of hemostatic treatment with BPA. Following administration of hemostatic agent, some patients with AHA shorten or even normalize APTT, which falsifies the results of subsequent laboratory tests and may lead to erroneous exclusion of AHA as the cause of hemorrhage. If a set of AHA confirmation tests cannot be performed at a local laboratory, platelet-poor plasma samples can be frozen to ≤ -20 °C for use in further diagnostics. According to the principles of good practice, coagulation tests should be performed on the samples of the same batch of material for unequivocal interpretation of results.

The laboratory diagnosis of AHA is mainly confirmed by the presence of the following in patient's plasma: isolated prolongation of activated partial thromboplastin time (APTT), circulating anticoagulant in the APTT mixing test (correction test), decreased FVIII activity, presence of FVIII inhibitor and exclusion of other related bleeding disorders, lupus anticoagulant (LA) or drug-induced inhibitors. The algorithm of AHA diagnosis is presented in Figure 1.

Hemostasis screening tests are routinely ordered and provide clinicians with important diagnostic and therapeutic guidance. In the diagnosis of AHA, the measurement of Activated Partial Thromboplastin Time (APTT) is of particular importance, as its prolongation is the first laboratory clue to AHA diagnosis. Platelet poor plasma is incubated for 3–5 minutes at 37 °C with phospholipids, contact activator, followed by calcium chloride addition. That initiates the intrinsic coagulation pathway and APTT is the time taken for a fibrin clot to form (normally about 27–35 seconds [5]). Sensitivity and specificity of the test are determined by the composition of the APTT reagent (type of activator and phospholipid mixture) as well as coagulation analyzer [6]. APTT is prolonged in factor XII, XI, IX VIII deficiency and to a lesser degree, in the deficiency of factors II, V, X and fibrinogen. In a person with AHA, laboratory tests typically show a 2–3-fold prolongation of activated partial thromboplastin time (APTT) at normal prothrombin time (PT), thrombin time (TT), closure time (CT) in PFA-100/-200® (platelet function analyzer), normal platelet count and the plasma fibrinogen level within the normal range [7]. Isolated prolongation of activated partial thromboplastin time (APTT) is determined in the presence of FVIII inhibitor (AHA, congenital HA) but also in

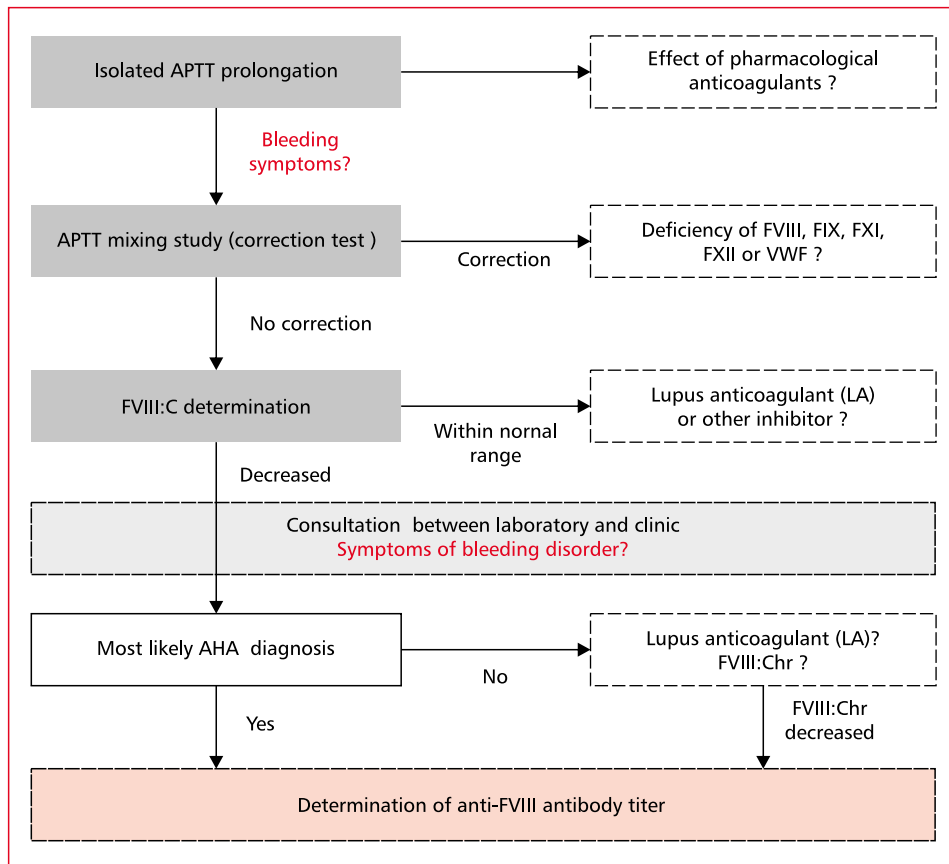


Figure 1. The diagnostic procedure algorithm for the diagnosis of AHA (modified from [8]). APTT — activated partial thromboplastin time; FVIII: C (factor VIII: C) — factor VIII/one-stage coagulation test; FVIII: Chr (factor VIII: Chr) — factor VIII/chromogenic test; AHA (acquired hemophilia A) — acquired hemophilia A, FIX (factor IX) — factor IX, FXI (factor XI) — factor XI, FXII Factor XII — factor XII, VWF — von Willebrand factor, LA (lupus anticoagulant) — lupus anticoagulant

deficiencies of FVIII (VWD, congenital HA), FIX (hemophilia B), FXI (hemophilia C), FXII (Hageman anomaly), high molecular weight kininogen (HMWK) or prekallikrein (PK) or in the presence of lupus anticoagulant (LA), anticoagulants (heparins, vitamin K antagonists (VKA), direct oral anticoagulants (Direct Oral Anticoagulants, DOAC), or inhibitor against another clotting factor. If prolonged aPTT is caused by the presence of unfractionated heparin in the blood sample, the thrombin time (TT) is significantly prolonged. Of particular importance for differentiation of AHA from the above-mentioned disorders is the presence of LA, which is not directed against the clotting factor but against phospholipids and does not trigger bleeding but predisposes to thrombosis. This requires careful analysis of the clinical picture of the patient, his medical history and laboratory outcome [6].

Extremely useful for distinguishing between a clotting factor deficiency as the cause of a pro-

longed APTT and the circulating anticoagulant (inhibitor) is the qualitative APTT mixing test (APTT correction test). A repeat APTT test is performed in a mixture of equal volume (1:1) patient plasma and normal plasma incubated for 1 h/37°C [5, 6]. The results of the correction test are properly interpreted only if patient’s baseline APTT is prolonged by at least 8–10 seconds above the upper limit of the reference range. The laboratory may have its own cut-off values depending on the measurement system [6]. According to definition, proper APTT correction occurs when the difference between APTT in tested plasma and mixture exceeds 50% of the difference between APTT of tested plasma and control. Normalization or significant shortening of APTT in mixing studies as compared to the patient’s baseline APTT is indicative of clotting factor deficiency. If the mixture fails to correct the APTT this is suggestive of the presence of circulating coagulation factor inhibitor [6]. Positive result is obtained in the case of FVIII

inhibitor (AHA) or LA as well as inhibitor against clotting factors other than FVIII. The APTT correction test can be performed in any laboratory that determines APTT, however, the availability of this test in multi-profile laboratories is still limited.

FVIII deficiency caused by anti-FVIII antibodies is diagnosed by determining FVIII activity in plasma. Routinely, the assay is performed with one-stage coagulation method (FVIII:C), less often — with the chromogenic test (FVIII:Chr). The FVIII:C assay is based on APTT measurement; diluted test plasma is mixed with FVIII deficient plasma, APTT reagent and calcium chloride. The clotting time of the mixture correlates with the FVIII activity in the tested system [6]. The FVIII:Chr assay consists of two stages, one is the production of FXa, the second is the hydrolysis of a specific chromogenic substrate. The intensity of the color correlates with the amount of FXa in the test plasma, which in turn is directly proportional to FVIII activity [5]. FVIII activity in healthy individuals is comprised in the range of 50–150 IU/dL (50–150% of the normal), while in AHA it is in the range of 0–20 IU/dL and in nearly 75% FVIII activity < 5 IU/dL [5, 8]. Unlike in congenital HA, the severity of bleeding symptoms does not depend on the level of FVIII in plasma. In laboratory practice, when AHA is suspected and FVIII:C activity reduced in a routine test, it is crucial to repeat the test in the patient's diluted plasma (e.g. 10×) to exclude interference of LA or other anticoagulants. For the one-stage coagulation method of FVIII:C determination, good practice recommends the use of APTT reagents with higher phospholipid content, thus insensitive to LA.

The primary goal in laboratory diagnostics of AHA is confirmation of the presence of FVIII inhibitor and measurement of its titer in the Bethesda Assay, fundamental for detection of neutralizing antibodies [9]. The subcommittee on Factor VIII, Factor IX of Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis (ISTH) has declared the Modified Nijmegen-Bethesda Assay (NBA) the “gold standard” for detecting antibodies against Factor VIII [9]. Currently, the NBA modification makes use of FVIII-deficient plasma (instead of veronal or imidazole buffer) to prepare serial dilutions of the patient's plasma or control plasma and to buffer standard human plasma with normal FVIII (SHP, Standard Human Plasma) [5]. This significantly contributes to better stability of protein concentration and pH in the mixtures during incubation and so the sensitivity and accuracy of the test is higher

[10]. The test also provided favorable conditions for FVIII neutralization through 2h incubation of the mixtures at 37°C. Following incubation, FVIII activity was determined in all tested samples and then compared with that in control mixtures to determine FVIII residual activity (RA) [9–12]. One Bethesda unit (BU) is defined as the amount of inhibitor that will neutralize 50% of FVIII:C in normal plasma [5, 11]. It is recommended to determine the inhibitor titer in samples with RA of 25% to 75% and in dilution where RA approximates 50% [9]. The inhibitor titer of ≥ 0.5 BU/ml is considered positive result, though some laboratories raised the threshold for positivity to ≥ 0.6 BU/ml [10]. The Bethesda test was developed to detect and quantify all FVIII antibodies in HA that exhibit linear type 1 kinetics. It is also useful for detecting FVIII inhibitors in AHA, but these often exhibit complex and non-linear type 2 kinetics and thus the concentration of these autoantibodies is more difficult to estimate. Initial thermal inactivation of the tested plasma (56°C/30 min), performed to eliminate too high residual FVIII activity, may improve the accuracy of the test [9]. The procedure is complex and so Bethesda assays are performed only in special reference laboratories of hemophilia treatment centers.

Commercial anti-FVIII ELISA tests were shown to be sensitive and specific for diagnosing AHA [8]. It worth noting however, that they may detect both FVIII neutralizing and non-neutralizing antibodies, depending on which kit is used. ELISA tests are particularly helpful for distinguishing anti-FVIII antibodies from LA. Moreover, determining isotopes of anti-FVIII antibodies may have prognostic value, as demonstrated in the case of anti-FVIII IgA [8].

A specific challenge to laboratory diagnosis of AHA is distinguishing of the type of “circulating anticoagulant” (FVIII inhibitor) detected in the APTT correction test from LA. This mostly refers to situations when the clinical symptoms of AHA are not obvious. The presence of LA can be excluded in the dilute Russell Viper Venom Time assay (dRVVT) [8]. Sporadically AHA coexists with LA, which significantly complicates the diagnosis due to false positive results of coagulation tests [13, 14]. The above may also apply to AHA patients treated with vitamin K antagonists (VKA), heparin or other anticoagulants. A chromogenic test is then used to determine FVIII activity and FVIII inhibitor titer. Unlike the routine coagulation tests, the chromogenic test is insensitive to the presence of LA or pharmacological anticoagulants [13, 14]. If

the dRVVT test is not available, a simple APTT test with reagents of higher phospholipid content and insensitive to LA can be used to distinguish LA from FVIII inhibitor [6, 8].

The diagnostic process of AHA combines the analysis of the clinical presentation and patient's history with the results of specialistic laboratory tests. Information on the medication that may affect the patient's coagulation system is also important. According to numerous experts, optimization of the diagnostic procedure involves close cooperation between the medical team and laboratory staff for efficient communication between both these professional groups. Such cooperation may optimize the diagnostic process and minimize the risk of delay in AHA diagnosis.

Conflict of interest: none declared

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