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Laboratory Misdiagnosis of von Willebrand Disease Caused by Preanalytical Issues: Sample Collection, Transportation, and Processing

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von Willebrand disease (VWD) is a genetic bleeding disorders caused by a deficiency of von Willebrand factor (VWF). Diagnosis or exclusion of VWD is not an easy task for most clinicians. These difficulties in diagnosis or exclusion of VWD may be due to preanalytic, analytical and postanalytic laboratory issues. Analytical systems to diagnose VWD may produce misleading results because of limitations in their dynamic range of measurement and low sensitivity. However, preanalytical issues such as sample collection, processing, and transportation affect the diagnosis of VWD profoundly. We will review here the common preanlytical issues that may impact the laboratory diagnosis of VWD.

Key words: Preanalytical, Variables, von Willebrand Disease (VWD), von Willebrand Factor (VWF)

REVIEW ARTICLE

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INTRODUCTION

Von Willebrand disease (VWD) is a congenital bleeding disorder caused by a deficiency of von Willebrand factor (VWF) and typically characterized by mild mucosal bleeding [1]. VWD may be caused by either a quantitative or a qualitative defect in VWF. The quantitative VWF defects are divided clinically into mild-moderate defects, known as type 1 VWD, and severe defects with undetectable VWF, known as type 3 VWD. The qualitative variants are known as type 2 VWD and divided further based upon the specific defect in VWF present. The current classification of the type 2 variants separates these into four groups [2].

The laboratory workup of VWD involves determining the level of VWF by both functional and antigenic methods. Per recommendations from the National Heart Lung and Blood Institute (NHLBI) that were developed by a working group in 2008, initial testing for VWD should include 1) VWF Ristocetin Cofactor assay, 2) VWF antigen assay, and 3) coagulation FVIII activity assay [3]. Second-tier specialized testing should include multimeric analysis, VWF collagen binding assay, ristocetin induced platelet aggregation (RIPA), platelet VWF assessments, genetic sequencing, FVIII binding assessment, and the VWF propeptide assay.

Despite these testing guidelines, several variables can affect the accuracy of VWF testing in general and this, in turn, may impact result interpretation and VWD diagnosis. Difficulties in achieving a correct diagnosis or exclusion of VWD might be due to analytical issues. Sometimes assays may generate a wrong result (i.e., due to analytical errors) or may have limitations in their dynamic range of measurement and/or their level of low analytical sensitivity, for example preventing

the correct identification of severe cases of VWD, which are characterized by very low factor levels. Less well recognized is the influence of preanalytical issues on the diagnosis of VWD [4]. During the pre-analytical phase, in case of hemostasis testing for patients with a positive history of hemorrhagic diathesis, certain steps regarding the preparation of patients and the execution of sampling (specimen collection, transportation, sample preparation, and storage) are of special importance. Therefore, this narrative review aims to provide an overview of some important preanalytical aspects such as sample collection, processing, and transportation that may affect the laboratory diagnosis of VWD.

SAMPLE COLLECTION

VWD studies tend to use blood anticoagulated with 3.2% sodium citrate, although 3.8% citrate concentrations are still in use in some centers. Most hemostasis researchers try to collect with minimal stasis or tourniquet use (to avoid hemoconcentration) and large-bore needles, or for platelet function to avoid platelet activation. Smaller one [23G] may be employed for pediatric use or difficult collections), as mentioned in several guidance publications [5-7]. There are no data to support the need for a discard tube for specialized hemostasis assays. However, due to a lack of sufficient evidence, the practice of drawing a discard tube should still be recommended [7]. Stress should be avoided under all circumstances (e.g., restless child) because stress increases acute phase proteins of which VWF, factor VIII, and fibrinogen are most important in particular in the workup for VWD. We also recognize the importance of proper tube fill volumes, with potential concern for both under-and over-filled tubes. Citrate tubes should contain 10% by volume of citrate anticoagulant, meaning 9 parts blood is added to 1 part anticoagulant. Under-filling tends to be most problematic, causing both dilution and over-citration ("over-anticoagulation") of plasma, leading to prolonged results for routine tests (e.g., PT and APTT), and false lowering of specific analytes (e.g., FVIII, FIX, VWF). This has the potential to cause misdiagnosis of VWD if analyte levels are close to cut-off or decision points (i.e., lower limit of the normal reference range, differentiating "normal" vs. "low"; typically around 50 U/dL, or "50% of normal"). Perhaps less well known/frequent, are effects of over-filling evacuated blood tubes (especially as available volumes facilitating over-filling are minimal for most blood tubes). The most likely effect here is to compromise adequate mixing of blood, potentially leading to sample clotting—either complete or partial [8]. Complete sample clotting leads to serum, with the potential loss of VWF through entrapment in the clot [9]. Partial sample clotting often leads to sample activation ("priming of coagulation"), which may shorten APTTs and artificially raise laboratory detected factor levels. Under-filled tubes may also lead to a false diagnosis of VWD or may exaggerate the perceived disorder. There are many situations where non-citrate anticoagulated tubes are collected for patients investigated for VWD. The best example is the EDTA tube for CBC, perhaps focusing on platelet counts as an alternative/complementary explanation of the bleeding disorder under investigation. EDTA samples are also used for VWF Multimer analysis and genetic testing [10].

Entrapment of VWF in clots may also lead to loss of VWF, preferentially high molecular weight (HMW) VWF, and thus misdiagnosis of VWD, especially type 2 [9]. Although workers in this field know such sample types to be inappropriate for standard VWD investigations, rejecting such samples when primary tubes were provided and thus visually inspected, problems may arise when testing laboratories are geographically distant from collection centers, with separation of "plasma" from centrifuged primary tubes, with (often frozen) material sent to test laboratories [11]. Once received frozen, the originating matrix is unclear, but can be determined if investigated. However, this adds cost and complexity and may not normally be executed.

The effect of blood hematocrit is another issue commonly discussed in sample collections and especially as related to pathology testing. The Clinical and Laboratory Standards Institute guideline on blood collection recommends undertaking citrate adjustments when hematocrit values are >0.55 [7].

Finally, all collected blood tubes should be labeled in front of the patient, using not less than two identifiers to avoid patient mix-ups, and attributing (analytically accurate) test results to the wrong patient.

SAMPLE TRANSPORTATION AND PROCESSING

Blood samples are usually need processed and transported to testing laboratories after blood collection. When possible, samples should be drawn directly in a laboratory. Immediately after drawing, whole blood should remain capped for transport both for safety reasons and to minimize loss of CO_2, which causes pH to increase, leading to prolongation of PT and/or aPTT [12]. Which comes first depends on geographical distances involved. For short distances, samples may be transported

whole, then centrifuged, then tested immediately or separated/frozen for subsequent testing. For long distances, samples may be centrifuged and then separated plasma transported (sometimes frozen) to testing laboratories [11]. Whole blood should be transported preferably upright in tube racks to avoid excessive agitation [7,9] and at "normal ambient" temperature (i.e., between 16 and 24°C). Extremes of temperature (low/refrigerated or high/"back of a van") should be avoided. Transport at high temperatures or even delayed transport at ambient temperature will lead to loss of labile factors and thus potentially to a false diagnosis of disease. Cold storage of citrated whole blood induces drastic time-dependent losses in VWF that contribute potential for misdiagnosis of VWD [13]. Refrigerated transport of whole blood should cause absorption of VWF (especially HMW VWF), and activation of platelets and FVII, thus leading to possible misidentification of VWD (especially as type 2); if transported this way, misidentification may occur in a normal sample, or from a type 1 VWD [14].

Whenever a delay in transport is expected, it might be advisable to perform local centrifugation and separation. Plasma is generally prepared by centrifugation of a whole blood sample. A temperature-controlled centrifuge is required for processing routine coagulation assays. Centrifugation should take place at room temperature (15–25°C) [7]. It is recommended to centrifuge the primary tube for coagulation testing at 1,500 g for no less than 15 min with a centrifuge brake set off. The centrifuge should be validated before use, every 6 months or after modifications, to assure that platelet-poor plasma (PPP) is achieved. Using relative centrifugal forces greater than 1,500 g is not recommended as this may induce platelet activation, hemolysis or other unwanted effects [7]. Alternate centrifugation, double centrifugation, protocols may be acceptable, providing residual platelet count is minimized and platelets are not activated by excessive centrifugal force. This low residual platelet count is perceived most important for lupus anticoagulant (LA) investigations, where double centrifugation protocols are commonly employed [15]. Depletion of platelets is especially important, should plasma be frozen/thawed, because residual platelets will be damaged and generate lysates quenching LA, thus potentially generating false-negative test results. For centrifugation, a swing-out bucket is recommended to minimize the remixing of plasma with platelets/erythrocytes, with resultant cell lysates on freeze/thawing. Filtration of plasma may cause loss of adhesive proteins (e.g., fibrinogen, VWF), leading to misdiagnosis of VWD [16], and is no longer encouraged, with double centrifugation now standard [15].

Whole blood assays should be performed within 4 hr after blood sampling and centrifugation should ideally be taken within 1 hr. Cold storage of citrated whole blood before centrifugation, by placing samples either in an ice bath or in refrigerated (2–8°C) storage, is no longer recommended. Improper storage of whole blood at cold temperature may cause VWF and factor VIII values to fall below normal reference threshold levels, which may potentially lead to a false suspicion of VWD due to inappropriate pre-analytical handling of blood [13,17]. When testing is not performed immediately, plasma can be frozen for later testing. Normal domestic freezers can be used for short-term storage (< 1 week), except if they are subject to cyclic freeze/thaw events (i.e. frost-free freezers). Otherwise, low-temperature freezers (≤ 70°C) should be used for long-term storage. Please refer to expert guidance on storage conditions [7,18]. Inappropriate thawing and mixing of plasma is another important issue. Different procedures may be used (i.e., gentle end-over-end inversions, blood tube rockers, vortex mixers), generating modest differences in clotting assays, including VWF [5,19-21]. Worse, no mixing leads to the clear potential for VWD misdiagnosis [20]. Therefore, laboratories should select one mixing procedure and then use standard operating procedures containing clear indications of the approved technique that should then always be followed for mixing thawed plasma. Mixing applies not only to test laboratories about to perform tests after samples are frozen/thawed but also to sample processing laboratories, which may thaw plasma post freezing to aliquot and dispatch to a distant laboratory.

CONCLUSIONS

The misleading results due to preanalytical issues can be minimized by the cooperation of laboratorians and clinicians. Clinicians should provide all relevant clinical information sufficiently for the laboratorians to adopt the proper techniques, and laboratorians should provide thorough post-analytical guidance on the test results including limitations and potential drawbacks and as well as for subsequent follow-up strategy.

Preanalytical issues arise uniquely for the specific reagents and analysis systems. These considerations imply that various preanalytical events may affect different test results, leading to complex test-panel patterns, and difficulty in interpretation, with subsequent potential for misdiagnosis of VWD. Standardizing efforts are needed to reduce the overall chances of the preanalytical phase, to generate high-quality test results and to

guarantee patient safety [5,22]. International external quality assessment schemes are also now starting to provide tools that cover the preanalytical issues of hemostasis testing.

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