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Technological advances in diagnostic testing for von Willebrand disease: new approaches and challenges

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SUMMARY

Diagnostic tests for von Willebrand disease (VWD) are important for the assessment of VWD, which is a commonly encountered bleeding disorder worldwide. Technical innovations have been applied to improve the precision and lower limit of detection of von Willebrand factor (VWF) assays, including the ristocetin cofactor activity assay (VWF:RCo) that uses the antibiotic ristocetin to induce plasma VWF binding to glycoprotein (GP) IbIXV on target platelets. VWF-collagen-binding assays, depending on the type of collagen used, can improve the detection of forms of VWD with high molecular weight VWF multimer loss, although the best method is debatable. A number of innovations have been applied to VWF:RCo (which is commonly performed on an aggregometer), including replacing the target platelets with immobilized GPIbα, and quantification by an enzyme-linked immunosorbent assay (ELISA), immunoturbidimetric, or chemiluminescent end-point. Some common polymorphisms in the VWF gene that do not cause bleeding are associated with falsely low VWF activity by ristocetin-dependent methods. To overcome the need for ristocetin, some new VWF activity assays use gain-of-function GPIbα mutants that bind VWF without the need for ristocetin, with an improved precision and lower limit of detection than measuring VWF:RCo by aggregometry. ELISA of VWF binding to mutated GPIbα shows promise as a method to identify gain-of-function defects from type 2B VWD. The performance characteristics of many new VWF activity assays suggest that the detection of VWD, and monitoring of VWD therapy, by clinical laboratories could be improved through adopting newer generation VWF assays.

INTRODUCTION

Laboratory tests are essential for the diagnosis and classification of von Willebrand disease (VWD) and the monitoring of VWD therapy [1–3]. The tests that have been widely used by diagnostic laboratories to assess von Willebrand factor (VWF) and classify VWF abnormalities have included assays of factor VIII coagulant activity, VWF antigen (VWF:Ag), and VWF activity [1, 2, 4–8]. In the developed world, a increas-

ing number of laboratories are using VWF assays that can be run on automated instruments [4–6, 8–11].

At present, VWF activity is most commonly measured by the ristocetin cofactor assay (VWF:RCo) that uses the antibiotic ristocetin as a cofactor to induce VWF binding to GPIbα, which results in platelet agglutination [2, 4, 6, 10]. Some laboratories supplement the measurement of VWF activity by VWF:RCo with collagen-binding assays (VWF:CB) to improve the detection of VWF defects associated with a loss of high molecular weight VWF multimers (HMWM) and/or impaired binding of VWF to vascular endothelial collagens [6, 10, 12, 13]. Methods that quantitate VWF levels using a monoclonal antibody to an epitope on VWF involved in binding GPIbα (e.g., HemosIL von Willebrand factor Activity assay) are not true activity assays and are not considered to be an adequate replacement for VWF:RCo, due to their poor agreement with methods that directly assess VWF binding to GPIbα [6, 14–17].

The lower reference interval limit for the ratio of VWF activity to VWF:Ag is commonly used to distinguish quantitative defects (i.e., type 1 VWD and more severe VWF deficiency due to type 3 VWD) from qualitative defects associated with types 2A, 2B, and 2M VWD, which impair VWF binding to the GPIbα component of platelet GPIbIXV [2, 6]. Assessments of ristocetin-induced platelet aggregation (RIPA), using concentrations of ristocetin that do agglutinate, and do not agglutinate, normal platelet-rich plasma, are used to distinguish gain-of-function defects from type 2B or platelet-type VWD, from loss of function defects due to type 2A or type 2M VWD [2, 6]. VWF multimer analyses are performed in few laboratories but remain useful to distinguish loss-of-function defects caused by type 2A VWD (from a loss of HMWM) from type 2M VWD (due to impaired VWF binding to GPIbα, which cannot be explained by a loss of HMWM, with or without reduced VWF:Ag levels) [2, 3, 6]. *VWF* sequence analysis is sometimes also performed, mainly at tertiary reference centers, to investigate and/or confirm VWD, including the forms of type 1 VWD (i.e., type 1C) associated with accelerated VWF clearance from plasma; type 2A, 2B, and 2M VWD; and type 2N VWD, which impairs factor VIII-VWF binding [2, 3, 6]. VWF propeptide (VWFpp) assays, which quantitate plasma levels of the N-terminal fragment of VWF that is released when mature VWF is generated, are performed by few centers [18–21]. The interpretation of VWFpp findings requires a comparison to the plasma level of mature VWF. The comparison of VWFpp to VWF:Ag shows promise as a method to detect defects associated with accelerated VWF clearance, including defects from type 1C VWD and acquired von Willebrand syndrome (AVWS) (e.g., due to an IgG paraprotein) [18–22].

This review highlights some of the recent technical innovations in the assessment of VWF levels and function, including new commercial, diagnostic assays.

DESIRABLE FEATURES OF ASSAYS FOR VON WILLEBRAND DISEASE TESTING

Assays with excellent sensitivity and precision, and a lower limit of detection that is adequate to distinguish between type 3 VWD and type 1 VWD, are desirable for VWD testing and monitoring [2, 6]. Among all VWF assays, VWF:RCo, performed on an aggregometer, has shown the poorest precision and the poorest sensitivity to low levels of VWF (Table 1) [5–8, 10, 23]. Other commonly used VWF activity assays have a better lower limit of detection (Table 1) [6, 10, 11, 23, 24]. The best lower limit of detection has been achieved with new methods that use a chemiluminescent end-point (Table 1) [25–27].

Presently, there is need for guidelines on the precision and limit of detection that are considered adequate for using VWF assays for clinical diagnostic purposes. With some methods, including VWF:RCo, it may be difficult or impossible to distinguish between type 1 VWD with VWF levels below 0.10 International units (IU)/mL from type 3 VWD, which could have impacts on treatment and management [2, 6]. VWF assay sensitivity, specificity, precision, and lower limit of detection are also important to distinguish between quantitative and qualitative abnormalities, and to decide whether additional tests (e.g., VWF:CB) should be performed to adequately detect qualitative defects [6, 10–12, 23, 24]. As VWF testing is also used to assess AVWS, which can present with qualitative and/or quantitative abnormalities, VWF diagnostic assays should be useful for an assessment of congenital and acquired forms of VWD [22].

In the developed world, VWF assays run on automated analyzers are highly preferred to aggregometry or ELISA based methods, and this is evident in sur-

Table 1. Limit of detection and imprecision of von Willebrand factor antigen and activity evaluated by platelet glycoprotein Ibα-dependent methods

Methodology	Imprecision (coefficient of variation, %)*	Limit of detection	Special considerations
von Willebrand factor antigen			
ELISA	10–20% [6]	0.02 IU/mL [6]	Not automated. Declining use.
Immunoturbidimetric assays	2.6–3.0% [27]	0.05 IU/mL [6] 0.02 IU/mL [11] 0.022 IU/mL [27]	
Chemiluminescent assays	7% [26] 3.9–5.3% [27]	0.005 IU/mL [26] 0.003 IU/mL [27]	Not available in some countries. Requires an ACL AcuStar instrument (Instrumentation Laboratory, Bedford MA)
Von Willebrand factor activity assays that use ristocetin as a cofactor			
Agglutination	20–40% [6, 25]	approximately 0.10 – 0.20 IU/mL [6, 11, 24, 29]	Limitations associated with using ristocetin. Declining use.
Immunoturbidimetric	3.8–6.2% [27]	0.03 IU/mL (modified assay) [11]	Limitations associated with using ristocetin
	<3.0% – <3.5% [40]	0.04 IU/mL [27]	
Chemiluminescent	7% [26] 4.2–6.9% [27]	0.002 IU/mL [26] 0.005 IU/mL [27]	Not available in some countries. Requires an ACL AcuStar instrument. Limitations associated with using ristocetin
VWF activity, evaluated with a gain-of-function glycoprotein Ibα mutant			
ELISA	10–20% [6]	0.02 IU/mL [6]	Not commercially available
Immunoturbidimetric	5.6% [29]	0.05 IU/mL [40] 0.04 IU/mL [29]	Not available in some countries. Gives lower results than VWF:RCo for some von Willebrand disease subjects [29]

*If imprecision was estimated for several samples, the value for the healthy control sample was used.

veys by proficiency testing organizations [4, 5, 8, 9, 23]. As few laboratories perform VWF multimer analysis, an assay that is technically challenging and not well standardized [4–6], technical innovations that overcome the need for VWF multimer analysis, or that simplify and improve multimer assay performance, would be welcomed by diagnostic laboratories.

Few studies have reported on the sensitivity and specificity of VWD assays as tests to detect VWD among subjects referred for bleeding disorder investigations, which is the main purpose of VWF assays performed in diagnostic laboratories, apart from therapy monitoring [1]. Some studies have assessed new assays for the detection of VWD and their ability to discriminate between quantitative and qualitative VWF abnormalities, using ratios of activity to antigen [15, 25, 28–30]. To avoid selection bias, some studies have evaluated a new method using consecutive samples referred for clinical diagnostic testing [29].

Before switching to a new method in diagnostic laboratories, it is important to consider the ability of the assay to detect VWF defects, and to distinguish quantitative from qualitative defects among prospectively evaluated patients that represent the full spectrum of subjects encountered in diagnostic practice. As VWF assays are used to diagnose and monitor VWD in adults and children, the evaluation of a new assay should include an assessment of pediatric and adult samples, including those drawn to evaluate VWD replacement or other therapies (e.g., desmopressin, intravenous gammaglobulin for acquired VWD due to an IgG paraprotein). Interference from other

variables, such as polymorphisms that affect some VWF test results but do not cause bleeding [31], and pre-examination errors [32] also need consideration.

Stringent regulatory requirements for diagnostic assay use in some developed countries influence which assays can be used for patient care, and whether laboratory developed tests and/or modifications to commercial methods are considered acceptable [33]. Many diagnostic laboratories prefer commercial assays as this reduces the resources required to validate modified procedures or 'in-house' methods. Nonetheless, there are gaps in the information provided by manufacturers of VWF assays. For example, VWF assay kits do not provide reference intervals for ratios of VWF activity/VWF:Ag, which are needed to distinguish qualitative from quantitative forms of VWD. Current guidelines for laboratories do not provide direction on how to establish such ratios, including the minimum number of healthy control samples required to establish appropriate cutoffs for VWF activity/VWF:Ag ratios.

IMPROVEMENTS IN METHODS FOR QUANTIFYING VON WILLEBRAND FACTOR ANTIGEN

Among VWF assays, VWF:Ag methods show the highest level of agreement and the best precision, based on the lowest, between-laboratory coefficient of variation in proficiency testing challenges [5, 7–9]. Not surprisingly, VWF:Ag assays performed on automated instruments are used far more commonly than ELISA in the developed world [4, 5, 8, 9]. Automated VWF:Ag assays that use an immunoturbidimetric end-point have a lower limit of detection of about 0.04-0.05 IU/mL, which is not an improvement compared with ELISA methods [24]. New assays that use a chemiluminescent end-point, such as the HemosIL AcuStar VWF Antigen (Instrumentation Laboratory, Bedford, MA, USA), currently have the lowest limit of detection (Table 1) [26, 27].

IMPROVEMENTS IN FUNCTIONAL METHODS FOR QUANTIFYING VON WILLEBRAND FACTOR ACTIVITY USING RISTOCETIN

A number of technical innovations have been applied to address the poor precision and inadequate lower limit of detection of VWF:RCo assays performed on an aggregometer [11, 14, 15, 17, 24, 25, 27, 31, 34–36]. All adaptations of VWF:RCo require ristocetin to induce VWF binding to the platelet VWF receptor GPIbIXV, whereas physiologic binding requires conformational changes in VWF induced by shear forces or VWF binding to the extracellular matrix [6, 31]. The first adaptations of VWF:RCo were the use of an automated instrument to quantitate the agglutination end-point [6]. While some VWF:RCo methods have been adapted for other platforms, such as flow cytometry [28, 34, 35], the most significant improvements in assay precision and limit of detection have come from using immobilized glycoprotein (GP) Ib (more specifically, GPIbα instead of platelets as the VWF capture), followed by quantitation by an ELISA, immunoturbidimetric, or chemiluminescent end-point on an automated instrument [6, 11, 14, 15, 17, 25, 27, 36]. The information on the precision and limits of determination, for modified VWF:RCo assays, compared with VWF:RCo performed on an aggregometer are summarized in Table 1. Proficiency testing exercises will be helpful to further assess the performance of new, automated, immunoturbidimetric, and chemiluminescent methods for measuring VWF activity with added ristocetin.

One limitation that is shared by all methods that use ristocetin to measure GPIb-dependent VWF function is that falsely low results are measured for subjects with *VWF* polymorphisms that reduce ristocetin-dependent VWF binding to GPIbα, without associated increases in bleeding [31, 37, 38]. The polymorphisms associated with falsely low VWF activity by ristocetin-dependent methods include p.(D1472H) and p.(P1467S) [31, 37, 38]. The p.(D1472H) polymorphism in *VWF* is the most common, and it results in a modest reduction (approximately 28% decrease) in VWF:RCo activity relative to VWF antigen among subjects with and without VWD [38]. This polymorphism does not affect VWF binding to an activation mutant of GPIbα that binds wild-type VWF without added ristocetin [38]. More striking, falsely low VWF activity by VWF:RCo has been reported for p.(P1467S) (approximately 92.5% decrease, based on data for three subjects), which is less common [31].

IMPROVEMENTS IN METHODS FOR QUANTIFYING VWF ACTIVITY: ASSAYS THAT DO NOT REQUIRE RISTOCETIN

There has been growing interest in measuring VWF activity without ristocetin, as this antibiotic is pro-

duced by a single manufacturer and it is recognized to have significant lot-to-lot variability. The strategies that have been applied to measure platelet-dependent VWF activity without ristocetin include flow (shear)-based methods (e.g., reference [39]) and ELISA or automated-binding assays that use gain-of-function GPIbα mutants to capture plasma VWF without the need for shear or matrix proteins [30, 31, 38].

There have been several studies of commercial, automated, immunoturbidimetric assays that use gain-of-function GPIbα mutants captured onto latex beads to measure VWF activity [29, 40]; information on their precision, and limit of detection, is summarized in Table 1. The Innovance® VWF Ac method has a better lower limit of detection, and better precision, than VWF:RCo estimated by aggregometry [29, 40]. Because World Health Organization (WHO) standards do not have an assigned a value for VWF activity by methods other than VWF:RCo, VWF:CB, and factor VIII, the Innovance® VWF Ac method uses VWF:RCo values for secondary standards, referenced against WHO standards. Bland–Altman analyses indicate that Innovance® VWF Ac results are, on average, about 0.06–0.07 IU/mL lower than VWF:RCo [29, 40]. Graf and colleagues reported that some patients with VWD have much lower levels measured by Innovance® VWF Ac than expected (based on bias estimates), whereas patients with a normal multimer distribution have findings that are consistent with bias estimates [29]. They observed that a type 2B VWD patient had much lower activity measured by the Innovance® VWF Ac than VWF:RCo method, both before and during VWF replacement therapy [29]. Furthermore, use of the Innovance® VWF Ac instead of VWF:RCo increased the number of cases considered to have qualitative defects of VWF, possibly from an increased sensitivity to qualitative defects in VWF-GPIbα binding, including the loss of HMWM [29].

Research on VWF:CB (which does not require ristocetin) indicates that, depending on the type of collagen used, these assays are helpful to identify patients with loss of HMWM and *VWF* mutations at sites involved in VWF binding to specific types of collagen [5, 6, 9, 10, 12, 13, 23, 41, 42]. However, the best methods are debatable as VWF:CB differ in sensitivity to HMWM loss [23]. Furthermore, the molecular mechanism underlying VWF binding to type VI collagen differs from the mechanisms that support VWF binding to types I and III collagen, and mutations affecting the binding to specific types of collagen have been described [6, 13, 42]. It is uncertain whether testing VWF:CB, using several types of vascular collagens, would reduce the proportion of patients considered to have an 'undefined' bleeding problem after an evaluation for VWD.

ALTERNATIVES TO RISTOCETIN-INDUCED PLATELET AGGREGOMETRY TO DETECT TYPE 2B VON WILLEBRAND DISEASE

There has been interest in alternatives to RIPA for identifying gain-of-function defects associated with type 2B VWD because RIPA requires rapid testing of freshly collected blood samples. This requirement precludes testing on frozen, shipped samples and means that patients must travel centers that offer RIPA, or undergo genetic testing for mutations associated with type 2B VWD. Newly developed ELISA, which use a gain-of-function GPIbα mutant as the capture for VWF, without added ristocetin, show promise in distinguishing type 2B from other forms of VWD [30]. While the immunoturbidimetric Innovance® VWF Ac similarly uses a gain-of-function GPIbα mutant to bind VWF and evaluate function, this method cannot be used to identify qualitative defects from type 2B VWD because type 2B, type 2A, and 2M VWD plasmas have similarly reduced VWF function by this assay [29]. It is unclear whether immunoturbidimetric methods could be modified to distinguish type 2B VWD from type 2A and 2M VWD.

TECHNICAL INNOVATIONS IN VON WILLEBRAND FACTOR MULTIMER ASSESSMENTS

Proficiency testing has demonstrated that errors are not uncommon among diagnostic laboratories that perform VWF multimer assays [5]. A number of technical innovations have been applied to the assessment of VWF multimer structure by expert research laboratories [43, 44]. It is possible that the increased sensitivity of newer VWF activity assays to the loss of HMWM could help identify which samples should be evaluated for an altered multimer distribution, as this is a feature of some congenital and acquired forms of VWD [22].

FUTURE DIRECTIONS AND CONCLUSIONS

In recent years, technical innovations in VWF assays used to quantitate VWF:Ag and VWF activity have led to significant improvements in the precision, lower limit of detection, and overall performance of VWF assays used for diagnostic and therapy monitoring purposes. Some of the innovations applied to measuring VWF activity have overcome the need to use ristocetin to measure VWF binding to its platelet receptor, GPIbα. Practice trends show an increasing adoption of methods that use automated platforms, particularly for immunoturbidimetric methods. The development of ELISA that can identify type 2B VWD gain-of-function abnormalities suggests that in the future, it may be possible to assess type 2B VWD without using RIPA. The implementation of improved VWF assays is likely to have positive impacts on the diagnosis and classification of VWD, and the monitoring of patient responses to therapy.

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