Lower Limit of Assay Sensitivity: An Under-recognised and Significant Problem in von Willebrand Disease Identification and Classification

EMMANUEL J FAVALORO, ROSLYN BONAR, KATHERINE MARSDEN

von Willebrand disease (VWD) is the most common inherited bleeding ailment, and is characterised by low levels of, or abnormal function in, the plasma protein von Willebrand factor (VWF). However, the laboratory testing process is problematic because of both the heterogeneity of VWD and the limitations in the tests used to identify reduced or abnormal VWF.

OBJECTIVE: This study reports on the lower levels of sensitivity for the different assays used in the diagnostic process for VWD and their significance in the diagnostic identification and classification of VWD.

METHODS: The RCPA Haematology QAP is an international external quality assurance (EQA) program that includes VWF/VWD testing within one of its special haemostasis modules. Over the past 10 years, over 50 samples have been distributed to participants, including five samples devoid of VWF and derived from either true Type 3 VWD patients or else from commercially purchased VWF deficient plasma. Samples were tested blind by study participants, who report back both numerical values (for VWF and Factor VIII:C) and an interpretation regarding whether or not VWD is suggested by laboratory findings, and if so, the probable VWD subtype.

RESULTS: Returned data indicates that the lower level of sensitivity (LLS) tends to be around 5-10U/dL for Factor VIII:C, VWF antigen (VWF:Ag), VWF collagen binding (VWF:CB), and VWF 'activity' (VWF:Act), but can reach 20U/dL or more for VWF ristocetin cofactor (VWF:RCo). There does not appear to be any improvement over the past decade despite ongoing automation of methodology, and indeed, automation does not seem to provide better LLS performance.

CONCLUSIONS: Limitations in the LLS of VWD testing have significant implications in terms of the identification and classification of an individual's VWD, given that these laboratory assays are used to identify VWD and help characterise functional VWF discordance, and that the majority of *severe* VWD subtypes have levels of VWF below 20U/dL.

Thus, laboratories will sometimes be unable to distinguish whether VWF deficient samples derive from Type 3 VWD or severe Type 1 VWD or even Type 2 VWD.

ABBREVIATIONS: ELISA = Enzyme Linked Immuno-sorbent Assay; EQA = external quality assurance; LIA = Latex Immuno-Assay; LLS = Lower limit of sensitivity; QAP = Quality Assurance Program; VWD = von Willebrand disease; VWF = von Willebrand Factor; VWF:Act = von Willebrand Factor 'Activity' (assay); VWF:Ag = von Willebrand Factor Antigen (assay); VWF:CB = von Willebrand Factor collagen binding (assay); VWF:RCo = von Willebrand Factor Ristocetin Cofactor (assay).

INDEX TERMS: von Willebrand disease; von Willebrand Factor, testing; VWD, diagnosis, classification, assay variables; VWF.

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Von Willebrand disease (VWD) is the most common inherited bleeding disorder. Although the calculated incidence varies according to the method used, most estimates are around 0.1-1% of the general population.[1] VWD is diagnosed following a clinical and physical review, with personal and familial evidence of (primarily mucocutaneous) bleeding, and confirmed by laboratory investigation.[2,3] The latter typically entails initial plasma testing (laboratory 'screening') of factor VIII coagulant (FVIII:C), and VWF protein ('antigen'; VWF:Ag) and VWF function, with this classically assessed using the ristocetin cofactor (VWF:RCo) assay. Some more recent international attention has focussed on the collagen binding assay (VWF:CB) and other putative VWF 'activity' assays, as possible replacements to VWF:RCo, or as supplementary tests of VWF function.[4] Supplementary laboratory testing may also be performed and include VWF multimers, ristocetin induced platelet agglutination (RIPA), and/or VWF-factor VIII binding (VWF:FVIIIB). [2,3]

Most simply defined, VWD is characterised by defective function in, and/or reduced levels of, VWF. Six types of VWD have been defined, comprising Type 1, Type 2 (with breakdown into 2A, 2B, 2M and 2N), and Type 3.[1-3] Types 1 and 3 are quantitative defects. Type 1 individuals produce low levels of otherwise functionally normal VWF, and VWF is generally absent in Type 3 VWD. In contrast, Type 2 VWD represents qualitative defects characterised by the presence of dysfunctional VWF, with the particular defect or dysfunction characterised within the subtype.

Many investigators[5-16] have recently reported on inconsistencies in laboratory diagnosis of VWD. Of particular note, in a recently published European study involving 14 VWD treatment centres,[7] 57 of 150 index cases originally identified as Type 1 VWD were later reconsidered on multimer or genetic testing as Type 2 by the authors (i.e., identified to have abnormal multimer patterns, and also a high prevalence of VWF gene mutations). In the Canadian Type 1 VWD study, 12 of 194 families were later reclassified as Type 2 VWD following data re-evaluation,[8] and subsequently, a further 10 index cases were found to have either loss of HMW VWF or abnormal multimer patterns, and a further 11 cases with normal multimers had low VWF:RCo/VWF:Ag ratios (<0.6).[9] In a UK study of 40 families, recruited through the national network of Comprehensive Care Haemophilia Centres, and diagnosed to have Type 1 VWD,[10] six families (13.6%) were re-diagnosed to have Type 2 VWD. Although such misidentifications may reflect a variety of issues, including local clinical interpretation of laboratory data, LLS could clearly be a contributing factor in misdiagnosis.

Within this context, and because of the move towards automation[3-6] and the recent availability of genetic studies with phenotypic correlates,[7-13] we re-evaluated the lower limit of sensitivity (LLS) for the most commonly applied tests. We had three main aims, to determine: (i) on an inter-laboratory basis, the LLS for these tests; (ii) whether there has been any improvement over time; and (iii) whether automation has improved LLS detection.

MATERIALS AND METHODS

The RCPA Haematology QAP is an international subscriber based external quality assurance (EQA) program that includes VWF/VWD testing within one of its special haemostasis modules. Currently, challenges are sent out as paired samples twice a year. Over the past 10 years, over 50 samples have been distributed to participants (Table 1), including five samples devoid of VWF and derived from either true Type 3 VWD patients or else from commercially purchased VWF deficient plasma. The true Type 3 VWD cases were provided for testing in despatches sent in 1998 and 1999, and commercial VWF deficient plasma was alternatively provided in subsequent challenges (2002, 2003, 2007) for several reasons, including ethical and safety. Samples are prepared by commercial lyophilization, and were despatched as either 1.0 or 0.5 ml aliquots. A minimum of 1.0 ml sample was provided to survey participants for each challenge.

On each occasion, samples were tested blind by study participants, who were asked to return for peer-based assessment: (i) numerical values for Factor VIII:C and for all VWF test parameters as performed in their laboratory, and (ii) an interpretation regarding whether or not VWD was suggested by their laboratory findings, and if so, the probable VWD subtype. While provision of numerical data is a mandatory requirement of participation, not all participants choose to provide an interpretation.

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RESULTS

Numerical results for the five samples tested over the analysis period for each test parameter as used by survey participants (i.e., VWF:Ag, VWF:RCo, VWF:CB, VWF:Act, and FVIII:C) are shown by year in Figure 1. In general, LLS tends to be around 5-10U/dL for FVIII:C, VWF:Ag, VWF:CB, and VWF:Act, but can reach 20U/dL or more for VWF:RCo. Results have been similar over the analysis period and data returned in 2007 does not show any evident improvement compared with earlier test periods. Figure 2 shows data separated according to whether a true Type 3 VWD sample was tested versus testing of a commercial VWF and FVIII:C deficient plasma for the major test types (i.e., VWF:Ag, VWF:RCo, VWF:CB, FVIII:C). Data shows broad similarity, although median FVIII:C is slightly lower using the commercial deficient plasma, and median VWF:CB is slightly higher. Figure 3 shows overall combined data, including that separated according to submethodology. VWF:Ag medians were similar for data obtained by enzyme-linked-immuno-sorbent (ELISA) methodology compared to latex-immuno-assay (LIA). Interestingly, the median VWF:RCo values were higher for automated agglutination assays compared to platelet aggregation procedures.

From the returned interpretative data provided by participants, we could determine (for data sets shown in Figures 1-3) that laboratories identified these samples as ‘Type 3 VWD’ on only 160/184 (87.0%) of total interpretation occasions, with severe Type 1 VWD identified on 16/184 (8.9%) occasions, and Type 2 VWD also occasionally identified and accounting for an overall error rate of around 13%.

DISCUSSION

These results have significant implications in terms of the correct identification and classification of an individual’s VWD, given that the core set of assays evaluated in this study (i.e., FVIII:C, VWF:Ag, VWF:RCo, and more recently and increasingly, VWF:CB and VWF:Act) are used in the initial patient workup to identify whether or not VWD is likely, and if so, to help characterise functional VWF discordance and hence provisionally characterise these VWD cases as either Type 1, 2 or 3. Moreover, it needs to be recognised that the majority of *severe* VWD subtypes have levels of VWF below 20U/dL. Thus, it is clear from this study that laboratories will sometimes be unable to distinguish whether truly VWF deficient samples actually derive from Type 3 VWD or severe Type 1. In addition, they will also occasionally misidentify VWF deficient samples as Type 2, since laboratories may identify a false functional discordance (e.g., VWF:Ag of 10U/dL and VWF:RCo of 0U/dL). Moreover, we should also recognise that this LLS issue will also lead to the occasional failure to identify VWF true functional discordance in true Type 2 VWD samples presenting with VWF <20U/dL (i.e., true Type 2 VWD samples may appear as Type 1).[3-6]

Indeed, this data may also understate the issue for VWF:RCo, as many laboratories report values to our EQA as ‘<20’ or similar, and these are normalised (to a midpoint value between this and 0U/dL) to permit data evaluation. There does not appear to be any improvement over the past decade despite ongoing automation of methodology (Figure

Table 1. Summary of the VWF/VWD surveys undertaken by the RCPA Hematology QAP and the year distributed

Year	Number of samples	Number of laboratories	Sample types: Normal	Equivocal	VWD type: 1	2A	2B	2M	2N	3	Other
1998	10	25	3	2	1	1	2			1	
2000	7	19	1	1	1		1	1	1	1	
2002	8	44	4	2		1				1	
2003	8	45	1	4	1	1				1	
2004	6	45	2	1	1						2
2005	4	49	2	1	1						
2006	4	53	1				3				
2007	4	55			2		1			1	
Totals:	*51*		*14*	*11*	*7*	*3*	*7*	*1*	*1*	*5**	*2*

*The subject of the current report.

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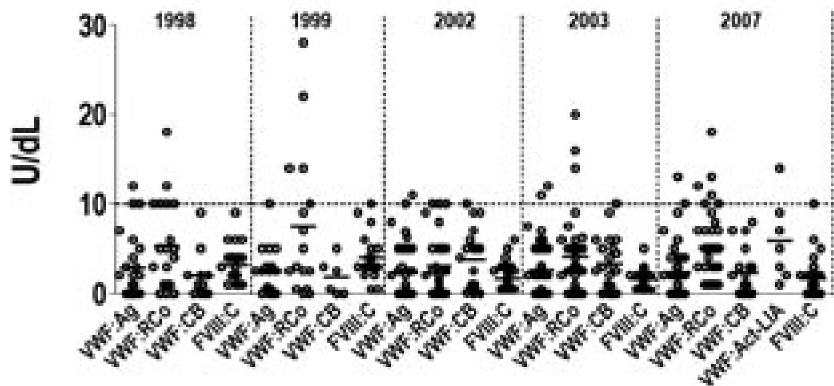
1), and indeed, automation does not provide for better LLS performance (Figure 3); this seems to be the case for both VWF:Ag and VWF:RCo.

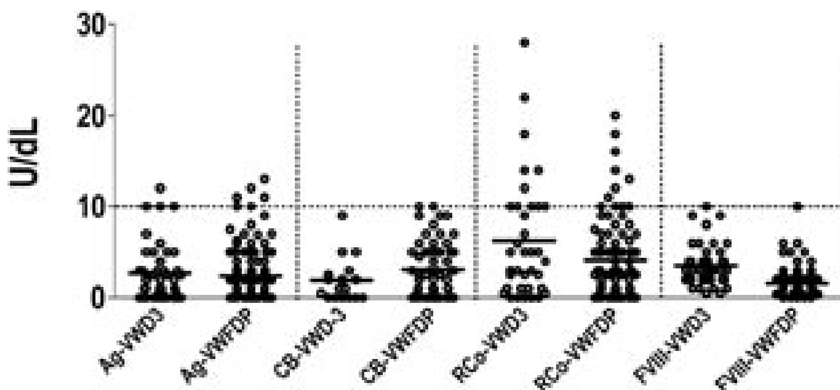
This data, related to potential misdiagnosis of Type 3 VWD (as Type 1 or Type 2 VWD), using laboratory testing on VWF deficient plasma, expands on previous reports from our EQA related to potential misdiagnosis of Type 2 VWD as Type 1 or Type 3 VWD, and potential misdiagnosis of Type 1 VWD as Type 2 or Type 3 VWD.[5,6]

Figure 1. Results of testing of either true Type 3 VWD cases (1998, 1999) or commercial VWF deficient plasma (2002, 2003, 2007) by participants of the RCPA QAP for FVIII:C, VWF:Ag, VWF:RCo, VWF:CB, and VWF:Act (by LIA)



Data shown (according to year of testing) as values reported by laboratories (y-axis; U/dL); results reported as '<' a given value are normalised to a midpoint between 0 and this value. Four gross outlier data points have been omitted from this data set (i.e., two VWF:RCo values reported as 87U/dL and 36U/dL, one VWF:Ag of 33U/dL, and one FVIII:C of 22U/dL.

Figure 2. Same data from Figure 1, but shown as composite data separated into true Type 3 VWD cases (VWD3) versus VWF deficient plasma (VWFDP)



There can be limitations to performance of such studies in an EQA setting. These include the likely criticism that the lyophilised samples provided to participants do not reflect real life settings of native (for example frozen) plasma. However, we perform extensive testing on these samples prior to despatch, including stability and homogeneity studies, and there is no evidence that sample presentation of lyophilised material adversely affects testing at laboratory sites. Another potential criticism would be that the volume provided (1.0ml) is insufficient to perform extensive (e.g., repeat confirmation) testing. Although a valid criticism, the volume provided is in fact similar to that provided to laboratories in the real world of referred testing, and it also needs to be remembered that repeat testing *per se* will often just lead to the same indeterminate value (e.g., '<20U/dL') for some test methodologies. In addition, other EQA providers[14-16] also report significant issues with assay variation and the laboratory identification and classification of VWD that would broadly be in support of our findings.

Another final criticism might be that the findings in this report just reflect the situation in a broad cross section of laboratories (i.e., both experienced and inexperienced), and that assessments of more experienced laboratories will not yield the same concerning findings. While this would best be addressed by a proper prospective study, a literature review of recent genetic VWD studies[7-13] indicates that diagnostic errors also occur within experienced VWD centres, and that a significant number of these could potentially be related to LLS issues.

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The additional reports cited in the introduction[7-13] relate to VWD treatment centres, and we would propose that LLS would likely have reflected at least a part explanation of diagnostic and classification errors in those studies. Of some considerable relevance to the current report, nearly half of all cases showing VWF mutations in these studies[7-13] have VWF values that were detected in the low assay detection range (i.e., <20U/dL). Some of the available data has been shown in Figure 4.

Figure 3. Same data from Figure 1, but shown as composite data for all testing cases separated by methodology (VWF:Ag ('Ag'); ELISA (E) vs LIA; VWF:RCo ('RCo'); platelet aggregometry ('agg') vs. automated agglutination ('auto'))

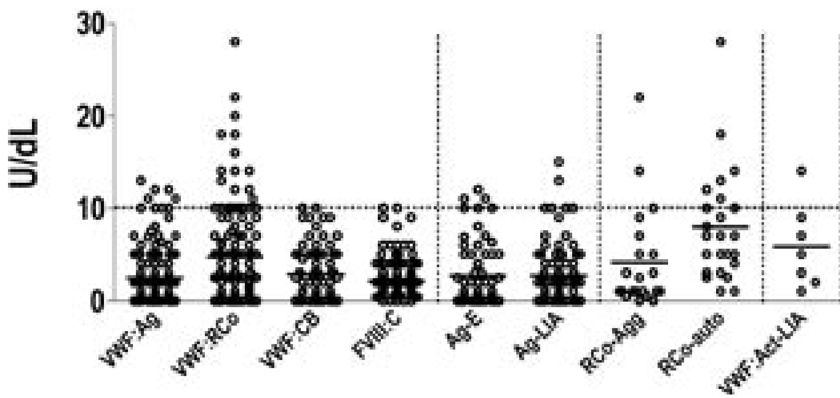
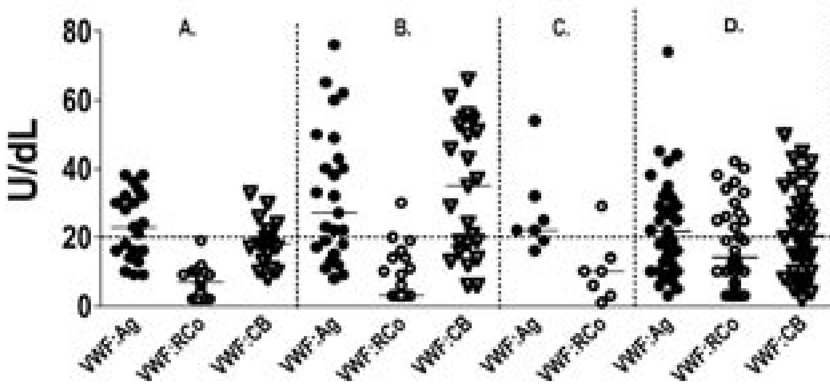


Figure 4. Summary of recently published phenotypic data from genetically confirmed cases of 'qualitative' VWF defects potentially fitting into a Type 2 VWD classification, and showing reported results, where available, for VWF:Ag, VWF:RCo, and VWF:CB



Sections A-D respectively indicate data from Penas and others,[11] Riddell and others,[12] James and others,[13] and Goodeve and others.[7]

RECOMMENDATIONS AND CONCLUSION

What the above issues mean in practice is that laboratories need to put several strategies into place in order to reduce the possibility of errors in VWD diagnosis because of LLS issues. We would summarise the main strategies as follows:

(i) Choose methodologies capable of improvements in detection at levels between 0-20U/dL.

(ii) Repeat VWF tests at least once using a fresh sample, for confirmation of previous findings (note that sometimes tests have to be repeated several times).

(iii) Use a comprehensive range of appropriate controls in laboratory assays; this is critical, but in our experience often overlooked by laboratories; in addition to a normal plasma and a mild Type 1 VWD-like control, laboratories are encouraged to also employ a Type 3 VWD-like and Type 2A VWD-like controls with all assays. All of these are available commercially or can otherwise be sourced. The Type 3 VWD-like control can be a VWF deficient plasma, and cryosupernatant (often available as a date-discarded blood banking product) can be used as a Type 2A-like control.

(iv) LLS detection issues can often be overcome by increasing the concentration, or lowering the dilution, of test plasma utilised in laboratory assays; ie plasma can be retested at dilutions that bring the derived test value to within ideal assay sensitive regions; typically around 20-100U/dL. For ELISA based assays, this may entail a 2-5x

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increase in plasma concentration. For automated assays, this feature is often available within reflexive testing.

(v) Perform as comprehensive a test panel as possible. For example, we have consistently reported that addition of VWF:CB to a core test panel of FVIII:C, VWF: Ag and VWF:RCo, substantially reduces VWD-diagnostic error rates.[3-6] Supplementary testing (e.g., VWF:multimers, RIPA, VWF:FVIIIB) should also be performed if required.

In conclusion, we report LLS data for the core laboratory assay panel typically used by most laboratories to provisionally identify, and then provisionally sub-classify, VWD (i.e., to characterise functional VWF discordance). LLS tends to be around 5-10U/dL for FVIII:C, VWF:Ag, VWF:CB, and VWF:Act, but 20U/dL or more for VWF: RCo. Automation of test procedures has not provided better LLS performance, nor does automation appear to protect laboratories from making diagnostic errors in VWD. These findings reflect serious diagnostic limitations, given that the vast majority of *severe* VWD subtypes have levels of VWF below 20U/dL. This problem most likely affects many VWD test laboratories, 'expert' and 'non-expert' alike. Finally, we detail several strategies to reduce the likelihood of diagnostic errors arising from this issue. Future multi-centre prospective investigations are warranted to validate the best diagnostic approaches.

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