

The use of an algorithm for the laboratory diagnosis of von Willebrand disease

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ABSTRACT

Von Willebrand Disease (vWD) is a bleeding disorder caused by either quantitative (type 1 and 3) or qualitative (type 2) defects of von Willebrand factor (vWF). No single test is available that provides appropriate information about the various functions of vWF and the laboratory diagnosis of vWD is based on a panel of tests that includes the measurement of factor VIII coagulant activity (VIII:C), vWF antigen (vWF:Ag), vWF activity as measured by ristocetin cofactor activity (vWFR:Co), vWF multimer analysis, ristocetin induced platelet agglutination (RIPA), the factor VIII binding assay of plasma vWF and the bleeding time. Due to the heterogeneity of vWF defects and the variables that interfere with vWF levels, a correct diagnosis of types and subtypes may sometimes be difficult but is very important for therapy. Furthermore, the Ristocetin Cofactor test and the RIPA test are based on platelet agglutination in reaction with the non-physiological antibiotic, ristocetin. These tests also have low sensitivity and are difficult to standardise. Therefore, several analyses (tests) are required to diagnose vWD and it is important to take the pitfalls that these tests are subject to in consideration in the diagnosis of vWD.

In this article, the laboratory diagnosis of vWD is presented on patients with type 1, 2A, 2B and 2M vWD. The diagnosis is done by using an algorithm that is proposed by the guidelines for diagnosis and treatment of vWD in Italy. The pitfalls in this diagnosis of vWD are outlined by 4 other patients.

Introduction

Von Willebrand Disease (vWD) is a bleeding disorder caused by a quantitative or qualitative defect of vWF. vWF is a high molecular weight glycoprotein that plays an essential part in the early phases of haemostasis by promoting platelet adhesion to the sub endothelium and platelet aggregation under high shear stress conditions [1]. vWF is also the carrier of factor VIII in plasma and a deficiency or abnormality of vWF also results in an impairment of blood coagulation. By the non-covalent interaction between vWF and factor VIII, factor VIII is protected against binding to membrane surfaces and to proteolytic attack by a variety of serine proteases, including activated protein C [2]. In the majority of cases, vWD is a congenital disease that is inherited in an autosomal dominant fashion. Patients with vWD may have a mild, moderate or severe bleeding tendency since childhood, usually proportional to the degree of the vWF defect. Inherited vWD has been subdivided into three types that reflect its pathophysiology. Types 1 and 3 vWD reflect respectively, the partial or virtually complete deficiency of vWF. Type 2 vWD is a defect that is subdivided into 4 subtypes (2A, 2B, 2M and 2N). Type 2A refers to variants with decreased platelet dependent function and is associated with the absence of high molecular weight multimers. Type 2B refers to variants with increased affinity for platelet glycoprotein 1b. Type 2M refers to variants with decreased platelet dependent function not caused by the absence of high molecular weight multimers and Type 2N to variants with markedly decreased affinity for factor VIII.

The spectrum and severity of vWD is wide, ranging from few, doubtful haemorrhagic symptoms to severe life-threatening bleeding episodes. This is due not only to the heterogeneous vWF gene which may impair its haemostatic function, but also to the influence exerted by other genes (e.g. those for ABO blood groups) [3]. In addition many acquired conditions, either physiologic (stress, pregnancy) or pathologic (inflammation), can induce fluctuations in vWF levels [3]. This, highly variable clinical picture and the presence of many different defects in the vWF molecule, complicate the diagnosis of vWD [3].

The guidelines for diagnosis and treatment of vWD in Italy propose the use of an algorithm (Fig 2). We adopted these guidelines in our Haematology Clinic and tested the usefulness thereof.

Materials and Methods

Subjects

Patients referred to the Haematology Clinic with a history of a bleeding tendency were screened for von Willebrand Disease.

Sample collection

Blood samples were collected into two Vacutainer tubes containing 0.105M sodium citrate in a ratio of 1:9 with blood. Platelet-poor plasma was prepared by centrifugation of whole blood at 2000 g for 20 minutes at room temperature. Samples were stored in polypropylene tubes at -70°C until analysed. All tests (except the vWF-multimer test) were done on original aliquots that were not previously thawed.

Screening tests

The platelet count was determined on the TECHNICON H1 blood cell analyser (Bayer Diagnostics, Germany). A bleeding time, prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined on

each patient with the STart 4 coagulation monitor, (Diagnostica Stago, Asnières, France). Furthermore, the blood group of each patient was determined. Plasma factor VIII levels were also determined on the ACL coagulation analyser (Instrumentation Laboratories, Italy).

VWF antigen (vWF:AG)

The vWF concentration in plasma was measured with an enzyme-linked immuno-adsorbent assay (ELISA).

Ristocetin cofactor assay (vWFR:CO)

This assay is done with the ristocetin cofactor assay kit from Helena Laboratories (France). Formalin-fixed washed platelets do not agglutinate in the presence of the antibiotic ristocetin unless normal plasma is added as a source of vWF. The agglutination follows a dose response curve that is dependent on the amount of plasma vWF added. The test was done on the Monitor IV Plus platelet aggregometer (Helena Laboratories, France). A standard curve of calibrated human plasma is used as the standard against which the patient's plasma is measured.

Ristocetin induced platelet agglutination (RIPA)

RIPA is measured by mixing different concentrations of ristocetin ranging from 0.2 to 2 mg/ml with increments of 0.1 mg/ml with the patient's platelet rich plasma (PRP) in an aggregometer. The results are expressed as the concentrations of ristocetin (mg/ml) able to induce 30% agglutination. Types 2A and 2M vWD show a low response to ristocetin, i.e. ristocetin concentrations more than 1.2 mg/ml are needed to induce 30% agglutination. An important exception is type 2B vWD in which there is a hyper responsiveness to ristocetin, due to a higher than normal affinity of vWF to platelet GPIb [5]. In these patients low ristocetin concentrations (0.2 to 0.8 mg/ml) are needed to induce 30% agglutination.

vWF multimers

The multimeric structure of vWF in plasma was determined by a highly sensitive and rapid method described by Krizek and Rick in 2000 [6]. This method utilises submerged horizontal agarose gel electrophoresis, followed by transfer of the vWF onto a polyvinylidene fluoride membrane, and immuno-localisation and luminographic visualisation of the vWF multimer pattern. This method distinguishes type 1 from type 2A and 2B vWD. Type 2A shows a total absence of the high and intermediate molecular weight multimers and type 2B shows an absence of only the high molecular weight multimers. All the other types or subtypes show a multimer pattern similar to that of normal plasma. Advantages to this method include rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of vWF and elimination of radioactivity. Figure 1 shows multimer patterns of normal plasma, type 1, and 2A and 2B vWD.

Factor VIII binding assay

The capacity of plasma vWF to bind exogenous FVIII was measured with an ELISA. A micro plate (Maxisorp, Nunc, Denmark) was coated by incubation for 2 days at 4°C with 2 µg/ml of rabbit polyclonal antihuman vWF (Dako, Denmark). After washing with Tris 50 mmol/l - NaCl 100 mmol/l, pH 8.0 (TBS) buffer containing 0.1% bovine serum albumin (BSA) and 0.05% Tween, the wells were saturated with TBS containing 3% BSA. Then 100 µl of serial dilutions of plasma from patients and normal pooled plasma were added and incubated overnight at 4°C. Each patient sample was tested

in six serial dilutions, the first being adjusted to 5% vWF antigen level. After removal of endogenous FVIII using 350 mmol/l CaCl₂ (10 min, twice), 70mU of recombinant FVIII were added to each well. After incubation for 2 hours at 37°C and washing, bound FVIII was quantified using 1 µg/ml of peroxidase-conjugated sheep polyclonal antihuman FVIII (Kordia, The Netherlands). After washing, immobilised vWF was measured using 0.1µg/ml of peroxidase-conjugated rabbit polyclonal anti-human vWF (Dako). The colour was developed by addition of OPD and the OD was read at 490nm. Two reference curves were established in parallel, one for the quantification of immobilised vWF and one for the quantification of bound rFVIII by diluting normal pooled plasma. For each plasma dilution, the values of bound rFVIII were plotted against the amount of immobilised vWF. The slopes of the obtained regression lines reflect binding capacity of vWF to FVIII.

Results

Diagnosis of vWD

The algorithm used for the laboratory diagnosis of vWD is outlined in Figure 2. A proportional reduction of both vWF:Ag and vWF:RCo with a RCo/Ag ratio >0.7 suggests type 1 vWD. If the RCo/Ag ratio <0.7, type 2 is diagnosed. Type 2B vWD can be identified in cases of an enhanced RIPA (<0.8mg/ml) while type 2A and 2M in a low RIPA (>1.2mg/ml). Multimeric analysis in plasma is necessary to distinguish between type 2A vWD (lack of largest and intermediate multimers) and type 2M vWD (all the multimers are present as in normal plasma). In type 1 vWD the ratio between factor VIII and vWF:Ag is always >1. When this ratio <1, type 2N vWD is suspected and this type of vWD can be confirmed by performing a factor VIII binding assay.

The results of patients who have been diagnosed with the different types and subtypes of vWD are given in table 1. Patient 1 has low vWF:Ag and vWF: RCo values with a RCo/Ag ratio of 0.74, therefore >0.7, which suggests type 1 vWD. The RIPA is normal and high molecular weight multimers are present. The ratio between factor VIII and vWF: Ag is also >1. Patient 2 has a very low RCo value with a RCo/Ag ratio of 0.44. This suggests type 2 vWD. The RIPA was decreased (30% agglutination occurs at 1.5 mg/ml ristocetin) and the high and intermediate vWF multimers were absent, which indicates type 2A vWD. The multimeric pattern of this patient is showed in figure 1. Patient 3 also has a RCo/Ag ratio <0.7 which suggest type 2 vWD. The RIPA is however increased (30% agglutination occurs at 0.6 mg/ml ristocetin) and the high molecular weight multimers are absent. This indicates a type 2B vWD. Patient 4 is also a type 2vWD, since the RCo/Ag ratio is 0.55. The RIPA is decreased and the multimer pattern is normal. This patient is therefore diagnosed as a type 2M vWD.

The results of 4 patients where the diagnosis of the types and subtypes of von Willebrand disease are not clear are outlined in table 2. Patient 5 has an RCo/Ag ratio <0.7 which suggest type 2 vWD. The multimers were normal and suggested a type 2B, but the RIPA, however, was normal. Other members of this patient's family were diagnosed with type 1 vWD. The RCo value (or vWF:Ag) could be wrong in this case. In patient 6 the RCo value was almost twice as much as that of the vWF:Ag. This indicates a "super" functional vWF that does not exist. All the other results of this patient were normal. Patient 7 indicated a type 2 vWD, because of a RCo/Ag ratio <0.7. The FVIII/Ag ratio was also discrepant and indicated a type 2N vWD. The RIPA was also normal that fits in with a type 2N vWD. The HMW multimers, however, were absent, which indicates a type 2A vWD. Therefore the RIPA and/or the factor VIII level seem to be wrong in this case. Patient 8 also indicates type 2 vWD since the RCo/Ag ratio <0.7. The RIPA is however normal and the HMW multimers were present. This indicates on the type 2M vWD, but the RIPA cannot be explained.

Discussion

We have outlined a systematic way to diagnose vWD that is recommended by the International Society on Thrombosis and Haemostasis. It is important to note that the RCo/Ag ratio is necessary to distinguish between type 1 and type 2 vWD. The RCo test however has a poor sensitivity (50%) (Scott et al., 1991), is difficult to standardise (Casonato et al., 1999), and lack physiological analogue. It is however still the standard method for measuring vWF activity that is approved by the Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH). The low sensitivity of the RCo assay makes diagnosis of vWD difficult as seen in patients 5 and 6. In patient 5 the family history and the results of the other tests indicate a type 1 vWD, but the RCo value was not proportionate to the Ag level. In patient 6 the RCo value was twice as much as the Ag level that indicates an over-active vWF that does not exist in vWD patients.

The RIPA test is necessary to distinguish between type 2B and type 2A vWD and the multimer pattern will indicate type 2A or 2M vWD. It is also important to do the RIPA test with ristocetin concentrations ranging from 0.2 mg/ml to 2 mg/ml with increments of 0.1 mg/ml. This will indicate at which concentrations maximum agglutination value of 30% occur. The RIPA test that is currently used by most laboratories in South Africa is done with only 3 ristocetin concentrations (0.5, 1.0 and 1.25 mg/ml). It is sometimes difficult to determine if the RIPA is enhanced or reduced on only 3 values. This was seen in patients 7 and 8 where the RIPA seems normal, but the HMW

multimers were absent in patient 7 and in patient 8, all other results indicate a type 2M vWD. Laffan et al. (2004) also found the RIPA normal in patients with an RCo value of less than 30%. And they found the RIPA only decreased in severe forms of vWD.

Multimeric analysis in plasma is necessary to distinguish between type 2A vWD (lack of the largest and intermediate multimers) and type 2M vWD (all multimers present). Our method includes rapid processing, simplicity of gel preparation, high sensitivity to low concentrations of vWF and elimination of radioactivity.

Type 2N vWD can be suspected in case of discrepant values of factor VIII. Thus, in case of factor VIII levels lower than the vWF:Ag, diagnosis of type 2N vWD should be confirmed by the factor VIII binding assay.

Since vWD is such a complex diseases to diagnose, this systematic diagnosis process makes the diagnosis of vWD more accurate, which is very important for the treatment of the disease. There are however pitfalls in this diagnosis process that are due to the limitations in sensitivity, reproducibility and interlaboratory variability of the agglutination-based RCo and RIPA tests (Favaloro et al., 1999).

Another functional assay that more laboratories are starting to use over the last 7 years is the collagen binding assay (CBA) of vWF (Favaloro et al., 2004). The CBA is based on the ability of the HMW multimers of vWF to preferentially bind collagen. This is an ELISA-based assay where dilutions of the patient's plasma is added to a collagen coated ELISA plate and the amount of bound vWF evaluated using an anti-HRP-conjugated vWF antibody. The values are expressed in U/dl, considering the optical density observed in the normal pooled plasma dilution as 100. This assay has been shown to be sensitive in the discrimination of Type 1 and types 2A and 2B vWD (Favaloro & Koutts, 1997). Casonato et al., 2001 demonstrated that the CBA was consistently more sensitive to large and intermediate vWF multimer representation than the RCo assay, since none of type 1 vWD patients studied showed CBA more decreased than the Ag levels. That was not the case with the RCo assay. The decreased values of the CBA in type 2A and 2B patients were more consistent than that of the RCo assay. The CBA is however insensitive to type 2M vWD patients.

In conclusion, it is crucial to use this systematic way to diagnose vWD. The CBA should be included as a useful diagnostic test in the profile of vWD diagnosis to counteract the pitfalls in the diagnosis of this disease.

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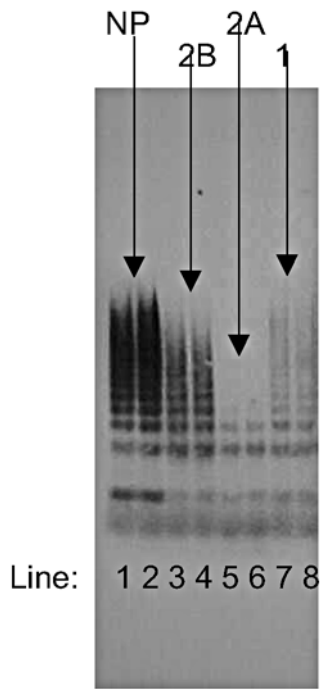


Figure 1
 The multimeric structure of vWF in normal plasma (NP) (lines 1 and 2) Type 2B vWD (lines 3 and 4), Type 2A vWD (lines 5 and 6) and type 1vWD (lines 7 and 8).

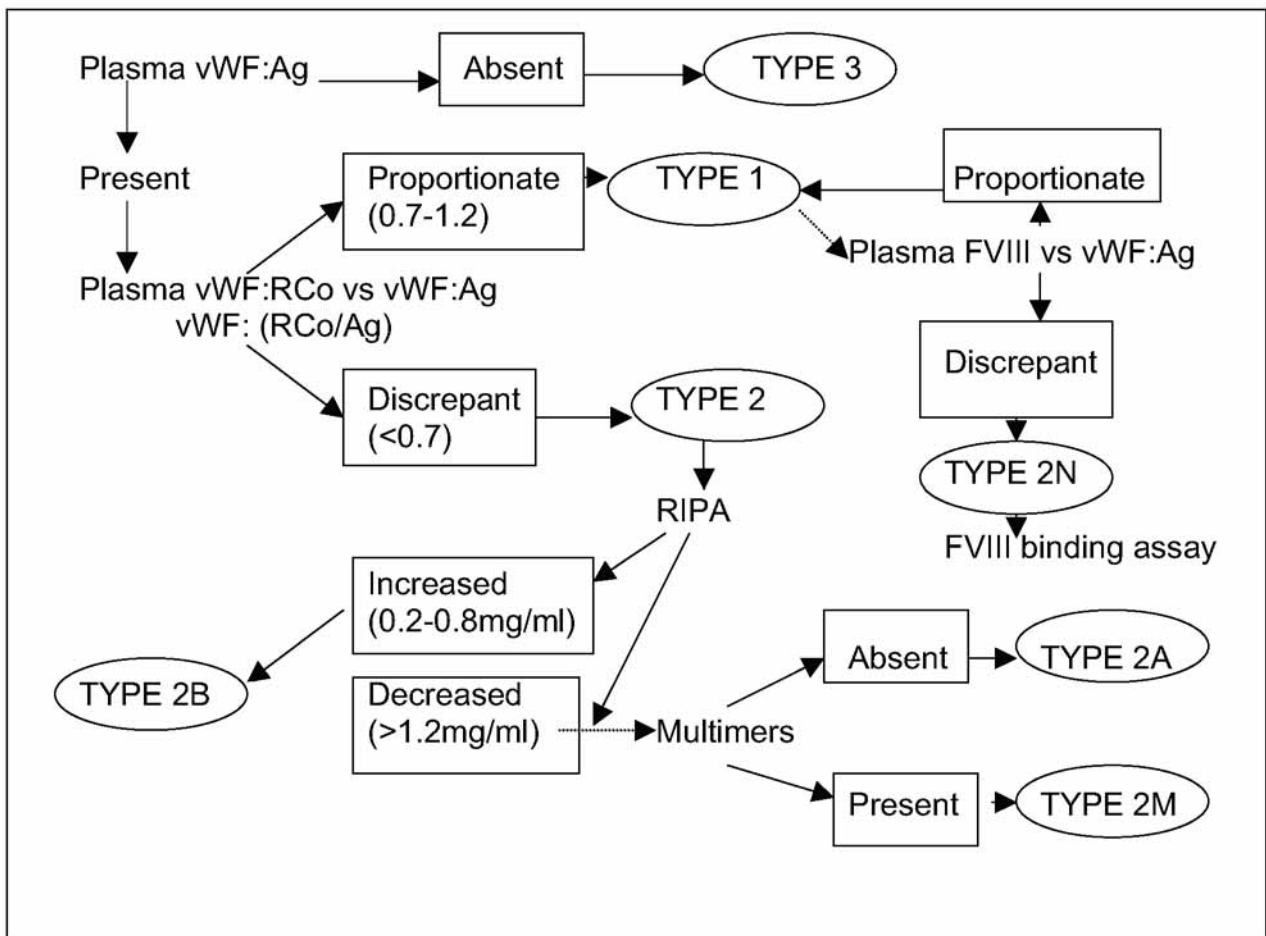


Figure 2
 Algorithm for the laboratory diagnosis of vWD

TABLE 1

Laboratory results of 4 patients with different type and subtypes of von Willebrand disease.

Tests	Patients			
	1	2	3	4
Factor VIII	123	57	49	77
vWF:Ag	54	54	42	82
vWF: RCo	40	24	22	45
RCo/Ag ratio	0.74	0.44	0.52	0.55
RIPA (Ristocetin conc.)	Normal (1.1mg/ml)	Decreased (1.5mg/ml)	Increased (0.6mg/ml)	Decreased (1.3mg/ml)
HMW Multimers	Present	Absent	Partially absent	Present
Diagnosed Type	Type 1	Type 2A	Type 2B	Type 2M

TABLE 2

Laboratory results of 4 patients where the diagnosis of the types and subtypes of von Willebrand disease are not clear.

Tests	Patients			
	5	6	7	8
Factor VIII	60	55	56	110
vWF:Ag	56	63	116	79
vWF: RCo	30	104	46	15
RCo/Ag ratio	0.53	1.65	0.4	0.18
RIPA (Ristocetin conc.)	Normal 1 mg/ml	Normal 1 mg/ml	Normal 0.9 mg/ml	Normal 1 mg/ml
HMW Multimers	Present	Present	Absent	Present
Diagnosed Type	Type 2M or 1?	Type 1?	Type 2A?	Type 2M?