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Full Length Article

Optimization of von Willebrand factor multimer analysis in vertical mini-gel electrophoresis systems: A rapid procedure

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ABSTRACT

Von Willebrand disease (VWD) is a common cause of bleeding worldwide. Analysis of von Willebrand factor (VWF) multimer distribution (VWF:MD) is essential to properly classify and treat different types of VWD, and it is performed using a SDS agarose gel electrophoresis followed by Western blotting, a handmade technique that demands days to be completed and requires skillful execution. Aiming both to facilitate gel production and to shorten the preparation time, we developed an uncomplicated technique to provide agility in the analysis of VWF:MD, so that it can be easily accomplished in the routine practice of hemostasis laboratories. On that account, we used a commercial vertical mini-gel electrophoresis system for SDS-PAGE and a semi-dry transfer system, which allowed us to analyze VWF:MD of various samples in a period shorter than 12 h. This technique differentiated VWF:MD in human and animal plasmas under normal, congenital and acquired (experimental envenomation by *Bothrops jararaca* snake) conditions. This optimized method is cheap, rapid, reproducible, easy to be performed, and uses electrophoresis and Western blotting systems available in most laboratories. All these advantages encourage hemostasis professionals to use it in their routine practices. In order to facilitate the setup and accomplishment of the whole procedure step by step, videos were appended to the article.

1. Introduction

Von Willebrand disease (VWD) is a prevalent disease [1], firstly described in 1924 (*apud* [2]), and it is due to a defect in the quantity and/or quality of von Willebrand factor (VWF) [3]. VWF is a very large glycoprotein, particularly involved in both platelet recruitment and adhesion to subendothelium under high shear stress, and the transport of blood coagulation factor VIII (FVIII) in circulation. VWF is present in different sizes in blood, varying from dimers of ca. 500 kDa to multimers of more than 10,000 kDa [4–6], and this heterogeneity in size is related to VWF activity, so that high molecular weight multimers (HMWM) are more functionally active than smaller ones [5,7,8].

In virtue of the complexity and heterogeneity of VWF [7,9], the phenotypic presentation of VWD shows a wide range of clinical manifestations and bleeding risks. Currently, congenital VWD is classified as type 1, 1C, 2A, 2B, 2M, 2N and 3. Patients with type 1 VWD have decreased VWF concentration with nearly normal VWF multimer distribution (VWF:MD), whereas type 3 VWD has a complete absence of VWF. Most patients with type 1C VWD have an increased VWF clearance, low VWF levels, an increased ratio of VWF propeptide to VWF:Ag, an enhanced response at 1 h after desmopressin administration (usually greater than 4-fold increase), and the presence of HMWM in some patients [10]. Qualitative defects in VWF are included in type 2 VWD, which is subdivided considering specific dysfunction or structural change in the VWF molecule. Thus, type 2A VWD has impaired platelet adhesion mediated by VWF with a selective deficiency of HMWM. Type 2B VWD shows VWF with enhanced affinity to platelet glycoprotein Ibα (GPIbα), and consequently patients have variable levels of thrombocytopenia, or even normal platelet counts, depending on the causative mutation. Type 2M VWD is characterized by diminished platelet adhesion dependent on VWF, along with normal levels of HMWM, and type 2N VWD has a marked decrease in binding of VWF to FVIII [5,11–14]. Furthermore, acquired von Willebrand syndrome (AVWS) has been described and it has been associated with lymphoproliferative and myeloproliferative disorders, neoplasias, aortic stenosis, and miscellaneous and immunological disorders [15–17].

Physical examination, family history, and a panel of laboratory tests are required for a correct diagnosis and treatment of VWD. Common

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practice laboratory tests evaluate plasma VWF levels and function, which serve as an important tool to classify VWD. These tests include a quantitative VWF assay (VWF:Ag), and assays that evaluate VWF function, e.g. the VWF:ristocetin cofactor (VWF:RCo) assay, the VWF:collagen binding capacity (VWF:CB) assay, the FVIII activity assay, and the ratios VWF:RCo/VWF:Ag, VWF:CB/VWF:Ag and FVIII/VWF:Ag [18].

Among the tests employed to diagnose VWD, the VWF:MD analysis is essential to provide an association between VWF structure and function. Particularly, it can evidence the absence of HMWM of VWF, which are associated with defective formation of platelet aggregates. In cases of abnormal in vivo VWF proteolysis, which promotes a loss of HMWM of VWF and a consequent accumulation of low molecular weight VWF multimers in circulation, the evaluation of VWF:MD is critical to the diagnosis [17,18]. The phenotype of VWF:MD is thereby considered a gold-standard test, which should be included in the diagnostic procedure for diagnosis and classification of VWD. However, given the technical difficulties of the assay, and the long duration to obtain results, this assay is currently performed only in specialized hemostasis centers or as a "second-line testing" in few routine hemostasis laboratories [12,18]. Moreover, the assay must be rigorously and carefully performed, because poor results can lead to erroneous classification of the VWD type [19].

Due to the high molecular weight nature of VWF, the evaluation of VWF:MD requires an electrophoretic run on agarose gels, which are more brittle than polyacrylamide ones, and a laborious procedure that usually requires various days to be completed [18,20]. Precast agarose gels can shorten the preparation and processing time [21]. Currently, a semi-automated equipment has been developed to carry out electrophoretic runs and VWF:MD analyses in one day (Hydragel 5 or Hydragel 11, https://www.sebia.com/en-EN/produits/hydragel-11-von-willebrand-multimers), but it cannot detect defects in the triplet structure of VWF or AVWS [22,23]. Inasmuch as most laboratories use commercial vertical electrophoresis systems for SDS-PAGE, we reasoned that they could also be used for VWF:MD analysis, and therefore could be implemented in the routine analysis of hemostasis.

On that account, we aimed to (a) obtain a sensitive and rapid method for VWF:MD analysis; (b) facilitate the preparation of agarose gels by employing a commercial mini-vertical gel system for SDS-PAGE; and (c) set up a procedure that could analyze various samples simultaneously. We report the use of a simplified technique to study VWD:MD in plasma samples from healthy donors and VWD carrier patients, as well as control and experimental rats and mice. The whole procedure can be completed in only 12 h, with conventional equipment found in most non-specialized laboratories.

2. Materials and methods

2.1. Ethics declaration

This study was approved by the Research Ethics Committee, and written informed consent was obtained from adult healthy human donors (CAAE 37958514.8.0000.0086, Ministry of Health, Brazil). Six plasma samples from type 2 VWD patients were kindly donated from Dr. Paula Ribeiro Villaça (Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil).

The use of male Wistar rats (250–300 g) and C57BL/6 mice (25–28 g) followed the guidelines from National Guidelines (Conselho Nacional de Controle de Experimentação Animal, CONCEA, Brazil), and was approved by the Institutional Animal Care and Use Committee in Instituto Butantan (CEUAIB 1117/2013). Male Wistar rats and male C57BL/6 mice were obtained from the Animal Facility, Instituto Butantan. Animals had free access to water and food, and were bred and maintained in a standardized environment, in rooms with defined flow of people, materials and supplies. In addition, they were continuously protected by health status barriers (barrier autoclave, HEPA air filtration system, differential pressure, etc.). To control ammonia in the environment, the exhausting system was kept at 15 to 20 air changes per hour at room level. The light cycle was defined as 12-h light: 12-h dark.

2.2. Reagents

Seakem® HGT(P) agarose was obtained from Lonza (Rockland, USA). Rabbit anti-human VWF antibody was purchased from Dako (A0082, Denmark), goat anti-rabbit IgG conjugated to Alexa Fluor® 647 was from Invitrogen (A21245, USA), and goat anti-rabbit IgG conjugated to peroxidase was from Sigma-Aldrich (A0545, USA). Lyophilized crude venom from a pool of adult specimens of *Bothrops jararaca* snake was obtained from the Laboratory of Herpetology, Instituto Butantan (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado, SisGen AF375C2). *Bothrops* antivenin was kindly donated from Instituto Butantan. All other reagents were of analytical grade or better.

2.3. Envenomation protocol and blood collection

Crude *B. jararaca* venom diluted in sterile saline was injected s.c. [1.6 mg/kg b.w [24]] in the dorsal region of male rats and male mice. Control rats and mice received saline. Blood samples were collected from the abdominal aorta of 2.5% isoflurane-anesthetized mice and rats at 6 and 24 h after treatment, respectively. Blood samples (9 volumes) were collected in 1 volume of 3.8% trisodium citrate solution containing protease inhibitors [25,26]. *Bothrops* antivenin (Instituto Butantan, lot 1305077) was used to neutralize the in vitro activity of *B. jararaca* venom in blood (1 volume of antivenin to 99 volumes of whole blood). Poor platelet plasma was obtained by centrifugation at 2500*g* for 15 min at room temperature, and the samples were maintained at room temperature during the whole process. Plasma aliquots were rapidly stored at −80 °C.

2.4. Animal plasma samples

The envenomed rat showed a plasma VWF:Ag level of 72 U/dL, a VWF:CB of 12.7 U/dL, a VWF:CB/VWF:Ag ratio of 0.18, and a platelet count of 434×10^9/L. The control rat had a VWF:Ag of 106 U/dL, a VWF:CB of 125 U/dL, a VWF:CB/VWF:Ag ratio of 1.18, and a platelet count of 1167×10^9/L. The envenomed mouse showed a VWF:Ag of 21 U/dL, and a platelet count of 260×10^9/L. The control mouse had a VWF:Ag of 116 U/dL, and a platelet count of 1118×10^9/L.

2.5. Electrophoresis

The protocol for discontinuous SDS-agarose electrophoresis was based on protocols described elsewhere [27,28]. All procedures of electrophoresis agarose, Western blotting, and development of nitrocellulose membranes are shown in videos (Supplementary material, Videos 1–4). The composition of solutions used in this protocol is detailed in Table 1.

One rectangular glass plate (10 × 10 cm, code SE262P-5) and one notched alumina plate (10 × 8 cm, code SE202N-10, GE Healthcare Life Sciences) were cleaned with alcohol and mounted within mini-gel casters, using 1.5-mm spacers. The mounted set was warmed at 37 °C in an incubator for at least 15 min prior to agarose solutions were poured. Running gel contained 1.6% (w/v) high gelling temperature agarose in running gel buffer, and stacking gel contained 0.8% (w/v) high gelling temperature agarose in stacking gel buffer. In order to dilute agarose, buffers were poured in 50-mL centrifuge tubes already containing the weighed agarose powder. Tubes were homogenized, and gel solutions were heated in a 10-liter capacity household microwave (1600 W), set at the maximum power, in the presence of a 200-mL recipient containing water as an energy buffer to absorb excessive microwave

Table 1
Solutions used for evaluation of VWF:MD using SDS-agarose gel electrophoresis and Western blotting.

Solutions	Composition
Anticoagulant for VWF:MD (Stable for at least 6 months at −80 °C)	3.8% trisodium citrate containing 50 mM Na_2EDTA, 60 mM N-ethylmaleimide, 5000 U/mL aprotinin, 10 mM AEBSF, and 280 μM leupeptin
Sample buffer (Stable for at least 6 months at 4 °C)	10 mM Tris, 1 mM Na_2EDTA, 2% SDS, 20% glycerol, and 2 mg/mL bromophenol blue (pH was not adjusted)
Running gel buffer (Stable for at least 1 month at 4 °C)	0.1 M Tris, 0.1 M glycine, and 0.4% (w/v) SDS, pH 8.8
Running gel (Stable for 4 h)	1.6% (w/v) of high gelling temperature agarose in running gel buffer
Stacking gel buffer (Stable for at least 1 month at 4 °C)	70 mM Tris, 4 mM Na_2EDTA, and 0.4% (w/v) SDS, pH 6.8
Stacking gel (Stable for 4 h)	0.8% (w/v) of high gelling temperature agarose in stacking gel buffer
Electrode buffer (Stable for at least 1 month at 4 °C)	50 mM Tris, 384 mM glycine, and 0.1% (w/v) SDS, pH 8.3 (not adjusted)
Transfer buffer (Stable for at least 6 months at 4 °C)	48 mM Tris, 39 mM glycine, 20% methanol, and 0.037% SDS, pH 9.2 (not adjusted)
Transfer buffer containing 1 mM 2-ME (Use immediately after 2-ME addition)	48 mM Tris, 39 mM glycine, 20% methanol, 0.037% SDS, pH 9.2 (not adjusted), and 1 mM 2-ME
Phosphate buffered saline (PBS) (Stable for 1 day at room temperature)	137 mM NaCl, 1.47 mM KH_2PO_4, 8 mM Na_2HPO_4, and 2.7 mM KCl, pH 7.4
Washing buffer (Stable for 1 day at room temperature)	PBS, pH 7.4, containing 0.1% Tween 20
Membrane blocking solution and incubation buffer for antibodies (Stable for 1 day at room temperature)	5% nonfat dry milk in washing buffer
Development solution for DAB staining (Use immediately, not stable)	5 mg of 3,3′-diaminobenzidine tetrahydrochloride dissolved in 10 mL of 0.1 M imidazole buffer (pH 7.0), and adding 125 μL of 0.2 M $CoCl_2$ and 3.4 μL of 30% H_2O_2

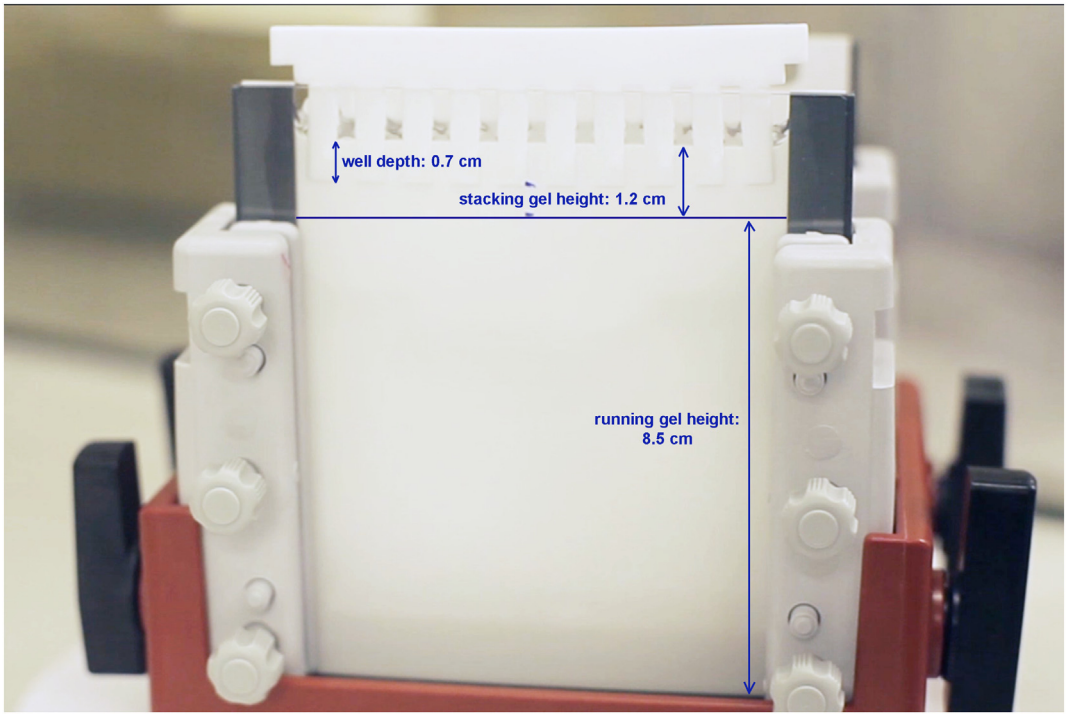


Fig. 1. Dimensions of the stacking gel, running gel and well depth for the current protocol, using a Mighty Small SE250/SE260 electrophoresis system (GE Healthcare Life Sciences).

energy. Care was taken to avoid boiling the buffer. Once agarose were completely dissolved, the agarose solutions were immediately transferred to the mounted plates in the gel caster using a 5-mL plastic pipette. In order to avoid agarose solidification during the transfer process, the tip of this pipette was pre-warmed using hot sterile water. Firstly, the pre-warmed 1.6% agarose solution (approximately 11 mL per set of mounted plates) was poured, until it reached 8.5 cm high from the bottom of the plates (Fig. 1). Secondly, after brief solidification on the bench of the 1.6% agarose solution, the pre-warmed stacking gel (approximately 2.5 mL per set of mounted plates) was poured until it reached 1.2 cm from the top of the running gel. Then a 1.5-mm comb (code SE211A-10-1.5, GE Healthcare Life Sciences), also pre-warmed in the incubator, was immediately inserted into the stacking gel, considering a depth of 0.7 cm for each well (Fig. 1). The mounted plates were maintained at room temperature until gel solidification, and then they were transferred and maintained

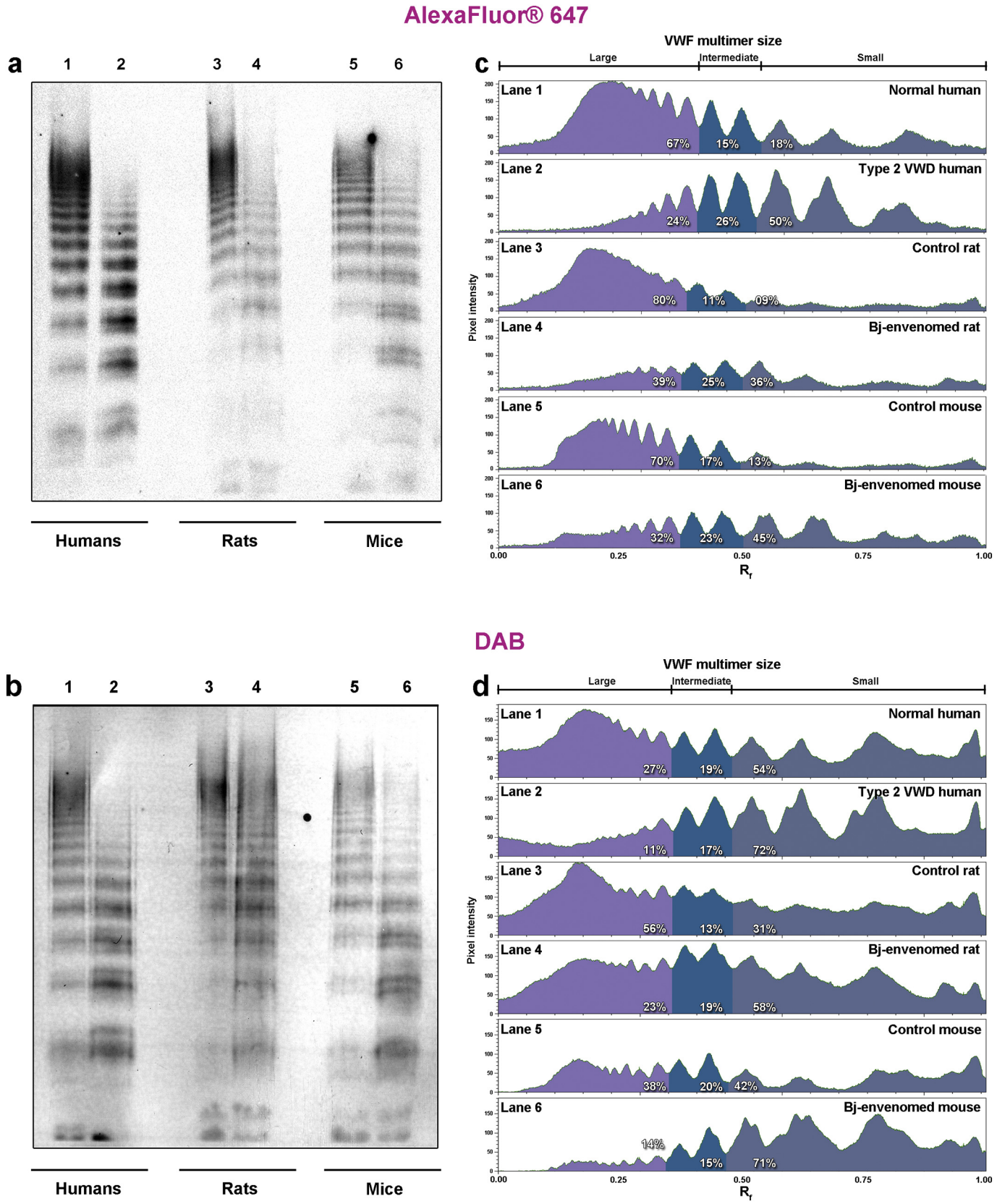


Fig. 2. VWF multimer distribution (VWF:MD) and densitometric analyses of plasmas from different species (human, rat, and mouse) after discontinuous SDS-agarose electrophoresis and blotting to nitrocellulose membranes. The dye front is at the bottom of the gel. VWF:MD was observed using antibodies conjugated with Alexa Fluor® 647 (a), or horseradish peroxidase (b) followed by staining with DAB. Samples: control plasmas from human (lane 1), rat (lane 3), and mouse (lane 5) individuals, showing normal VWF:MD; plasma sample from human patient with type 2 VWD (lane 2), showing lack of HMWM; plasma samples from a rat (lane 4) and a mouse (lane 6) injected with *B. jararaca* snake venom, depicting loss of HMWM, suggestive of acquired VWD. Densitometric analyses of lanes 1–6 stained by Alexa Fluor® 647 (c) or DAB (d) are shown in right side of the respective membrane images, and were obtained in TotalLab TL100 software. Percentage values of multimer groups are shown inside the respective areas in densitometric graphs. The final concentration of VWF:Ag in each lane was adjusted to a better observation of bands.

at 4 °C for 30 min. Thereafter, the plates were removed and placed in a precooled mini-vertical gel electrophoresis chamber (Mighty Small SE250/SE260, GE Healthcare Life Sciences), connected to a thermostatic circulator (MultiTemp III, GE Healthcare Life Sciences) maintained at 9 °C during the whole electrophoresis procedure. Refrigerated electrode buffer was placed in the inferior and superior cameras of the electrophoresis chamber, which at this point was already cooled at 9 °C. The room temperature was set to 18 °C to help reducing temperature variation during the electrophoresis run, since we observed, as pointed out elsewhere [29], that a minimal variation in gel temperature can interfere in band definition.

Plasma samples were diluted in sample buffer, taking into account the assayed VWF:Ag levels, so that they reached a final concentration of about 10 U/dL in all samples. VWF:Ag levels were determined by ELISA [30]. Following a preincubation at 60 °C for 30 min in a dry bath heater, samples (10 μL) were immediately loaded into the gel wells. To minimize artefacts, plasma preparation should be carried out immediately before submitting samples to electrophoresis. Following sample loading, the electrophoresis chamber was set up and wires were plugged into PowerPac Power Supplies (Bio-Rad), and the electrophoresis was run at 10 mA per gel. It took approximately 2 h to the tracking dye (bromophenol blue) exits the gel.

2.6. Blotting

Electrophoresis plates were then separated according to the manufacturer's recommendations, and the running gel was soaked in transfer buffer containing 1 mM 2-mercaptoethanol (2-ME) [31], and incubated for 30 min at room temperature under homogenization. Thereafter, prior to initiate protein transfer, the gel was washed using transfer buffer without 2-ME for 15 min at room temperature. Proteins were transferred onto 0.2-μm pore nitrocellulose membranes (code 162-0112, Bio-Rad), which had been previously hydrated in distilled water for 1–2 min and maintained in transfer buffer until use. Extra thick blot absorbent filter paper (Bio-Rad) was also presoaked in transfer buffer for 10 min before use, and employed to maintain gels in position. A gel sandwich was mounted, beginning with a filter paper, followed by the membrane and the electrophoretic gel, and finishing with another filter paper. Care was taken to remove all air bubbles from each step during the mounting of the sandwich gel. Protein transfer was carried out using a semi-dry transfer system (Trans-Blot SD, Bio-Rad) set at 23 V for 30 min [32]. Once the transfer was finished, the membranes were dried in an incubator (37 °C) to fix proteins. If necessary, the procedure may be stopped at this point, by freezing the membranes, so that they can be developed later. Once the membranes are dry, they should be rehydrated with distilled water for the next steps.

2.7. Membrane development

Initially, non-specific sites on nitrocellulose membranes were blocked by incubation with 5% nonfat dry milk in washing buffer for 1 h at room temperature under homogenization. An alternative procedure for this step is an overnight incubation at 4 °C. The membrane was then washed three times of 3 min each, under homogenization at room temperature, using washing buffer. Then, the membrane was firstly incubated with the primary antibody (rabbit anti-human VWF antibody, diluted 1:5000 in incubation buffer) for 2 h at room temperature, under homogenization. Following three washings of 5 min each, the membrane was incubated with the secondary antibody (goat anti-rabbit IgG conjugated to Alexa Fluor® 647, diluted 1:5000 in incubation buffer) for 1 h at room temperature under homogenization. Finally, the membrane was washed three times 5 min each, and the fluorescence of bands was detected in a ChemiDoc™ imaging system (Bio-Rad) using the Image Lab 5.2.1 software (Bio-Rad) set for a 647-nm filter. Alternatively, if fluorescence detection is not available, membranes may be incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:5000 in incubation buffer) and developed with chemiluminescence substrate [33] or 3,3′-diaminobenzidine tetrahydrochloride (DAB) [34]. Images from membranes stained with DAB were acquired and analyzed in TotalLab TL100 software (USA) [24].

2.8. Densitometry

A detailed quantification of different multimer size [35] should be undertaken by densitometric analysis. Briefly, densitometric analysis was carried out on captured blotting images (Fig. 2a, b) in TotalLab TL100 software (USA). To individualize the triplets for densitometric analysis, the triplet limits were set at the optical density valley between the adjacent faint side bands of the multimer central band (for small and intermediate multimers) or between 2 central bands (for HMWM) [35]. For sake of comparison between samples, from the end to the top of electrophoresis run, multimers were grouped according to their molecular size: peaks 1 to 4 were grouped as low molecular weight (LMW) multimers, peaks 5 and 6 as intermediate multimers, and peaks higher than 7 were grouped as HMWM (Fig. 2c, d). HMWM bands were grouped because the multimer band resolution could not provide enough discrimination to segregate them. The percentages of the optical density values of multimer groups in comparison with the total integrated area of each lane were reported, and used for comparison between samples and staining procedures (Fig. 2c, d).

3. Results

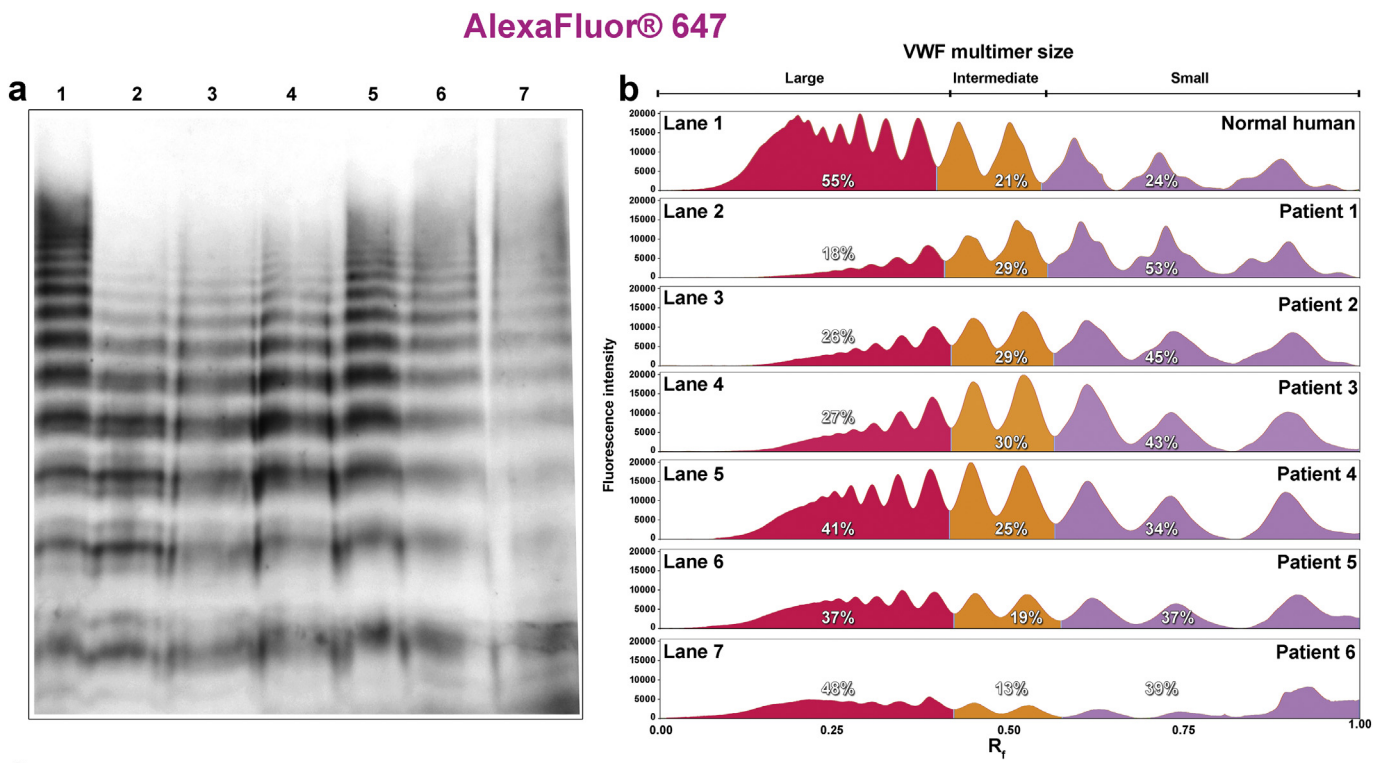
3.1. Improvements in agarose gel electrophoresis and VWF multimer blotting

Electrophoresis is a technique widely used to study the mobility of proteins based on their charge and/or size, and since the first use of SDS-agarose gel to study VWF:MD [36–38], several modifications have been made to improve multimer segregation and to facilitate the procedure [39].

In order to achieve VWF:MD analysis in a period of 12 h, we have optimized earlier techniques [27,28], so that the procedure described herein uses a vertical mini-gel electrophoresis chamber and a semi-dry protein transfer unit. In virtue of the small size of the running gel, 8.5-cm-long, the refrigeration of the electrophoresis chamber was combined with a high current setting, in order to achieve a better resolution of multimer bands. The electrode buffer was also refrigerated before and during its use, avoiding temperature fluctuation in gels [29]. Additionally, samples were collected using an anticoagulant containing protease inhibitors, and the stored plasma was unfrozen only one time before use. We also noticed that the storage of plasma in sample buffer impaired the analysis. The denaturation temperature of samples in SDS is also an important step to achieve well defined bands. In our experience, a prolonged incubation at a lower temperature (30 min at 60 °C) provided better results than a short incubation at a higher temperature (7 min at 100 °C). Furthermore, a semi-dry system for transfer of proteins was used. This type of transfer is not commonly used for VWF:MD analysis due to the large size of multimers, which are less easily blotted onto nitrocellulose membranes [31]. However, to depolymerize VWF in situ and to promote a superior transfer of HMWM to nitrocellulose membranes, the gel was incubated in transfer buffer containing 2-ME immediately after electrophoresis [31,40].

Another modification to improve band resolution was the use of fluorescence probes, which show a higher dynamic and linear range than chemiluminescence [41] and a higher sensitivity than colorimetric staining [42]. The use of Alexa Fluor® 647 showed a more intense signal than DAB staining, demonstrating that fluorescence is preferred for VWF:MD analysis. However, the colorimetric staining can be used for qualitative determination of VWF:MD when fluorescence detectors are not available.

In regard to costs, the procedure can be considered cheap, as each sample analysis cost US$ 3.20, regardless the methodology used to



c

Individuals	VWF:Ag (U/dL)	VWF:RCo (U/dL)	VWF:RCo/VWF:Ag ratio (U/dL)	FVIII (U/dL)	FVIII/VWF:Ag (U/dL)	Platelet count (×10⁹/L)	VWF:CB (U/dL)	Bleeding history
Normal control	122	NA	NA	NA	NA	323	NA	Absent
Patient 1	97	1	0.01	123	1.27	42	46	NA
Patient 2	45	19	0.42	38	0.84	181	NA	Mild epistaxis
Patient 3	47	22	0.47	35	0.74	NA	NA	NA
Patient 4	78	19	0.24	52	0.60	593	NA	NA
Patient 5	31	6	0.19	52	1.67	293	NA	Oral cavity, gastric, melena, anemia
Patient 6	25	7	0.28	28	1.12	277	NA	Mucocutaneous, tooth extraction, menorrhagia, anemia

NA- not available

Fig. 3. (a) VWF multimer distribution (VWF:MD) and (b) densitometric analyses of plasmas from a normal individual (lane 1) and patients 1–6 (lanes 2–7) with type 2 VWD, using the protocol described herein. (c) Laboratory and clinical findings from these individuals. VWF:MD was observed using antibodies conjugated with Alexa Fluor® 647. Plasma samples from patients 1–3 lacked HMWM (lanes 2–4), but not from patients 4–6 (lanes 5–7). Densitometric analyses of lanes 1–7 are shown in right side of the respective membrane image, and were obtained in Image Lab 5.2.1 software. Percentage values of multimer groups are shown inside the respective areas in densitometric graphs. The final concentration of VWF:Ag in each lane was adjusted to a better observation of bands.

develop nitrocellulose membranes (fluorescence, chemiluminescence or DAB). This value takes into consideration exclusively the prices of consumables (reagents, nitrocellulose membranes, blotting papers, and disposable plasticware), excluding equipment and labor costs. Interestingly, 40% of the costs were due to protease inhibitors used in the anticoagulant for VWF:MD (Table 1).

3.2. VWF:MD in plasma samples from congenital VWD or AVWS

Using the modifications described above, no loss of sensitivity in multimer band detection was observed. A great number of multimer bands, at least 11 distinct peaks in normal plasmas, independently of the species, as well as the triplet VWF structure [43] could be efficiently distinguished (Fig. 2). In fact, the discrimination of the VWF triplet structure could also be used for the diagnosis of defective multimerization in some patients with type 2 VWD [44–46]. Results from dozens of samples from animals and normal human plasmas showed reproducible results (cf. VWF:MD and densitometric analyses from normal plasma and patient 1 in Figs. 2a, b and 3a, b). Once the whole procedure has been established, it showed to be reproducible by different operators; for example, Figs. 2 and 3 have been obtained and analyzed by different operators (CMT and ATAS, respectively).

Membranes developed using Alexa Fluor® 647 (Fig. 2a) gave clearer images, low background, and HMWM peaks were more easily discriminated by densitometric analysis (Fig. 2c). On the other hand, in membranes stained by DAB, the background was higher, and the HMWM and intermediate multimer peaks were not quite discriminated (Fig. 2b, d). However, triplet structures were better discriminated by DAB (Fig. 2b) and by the respective densitometric analyses of LMW multimers (Fig. 2d) than by Alexa Fluor® 647 (cf. Fig. 2a, c). More

coherent results with the clinical and experimental procedures were noticed by Alexa Fluor® 647, since, as shown in Fig. 2c, d, the percentage values for bands stained with Alexa Fluor® 647 and DAB did not match sufficiently well. In general, the percentage values of optical density for the HMWM peaks from membranes stained with DAB were lower than those stained with Alexa Fluor® 647, and, on the other hand, were higher for LMW peaks (Fig. 2c, d), suggesting that the high background of DAB interfered in the detection of HMWM.

Fig. 3 depicts the VWD:MD (Fig. 3a), densitometric analyses (Fig. 3b), and the laboratory and bleeding history (Fig. 3c) available from a healthy donor and type 2 VWD patients. By evaluating the VWF:MD and other laboratory VWF parameters (the disproportional low VWF:RCo in comparison to VWF:Ag, FVIII levels and FVIII/VWF:Ag ratio) from six patients, they could be classified as type 2 VWD. The results presented herein are in agreement with those published elsewhere [47]. In patient 1, the condition could be classified as subtype 2B or 2A VWD (Figs. 2a, b, 3). The absence of HMWM in type 2A VWD and in most cases of type 2B VWD does not accurately distinguish them [48], but once patient 1 manifested thrombocytopenia, the diagnosis of type 2B VWD would be favored. Nonetheless, the hyperactive phenotype provided by mutations in platelet GPIbα could also lead to a very similar picture to VWD type 2B [49,50]. Patient 2 and 3 could be classified as type 2A VWD. Patient 4 – who showed a high platelet count, reduced levels of VWF:Ag, a low VWF:RCo/VWF:Ag ratio and a mild loss of HMWM – could be tentatively classified as type 2M VWD. The pattern of VWD:MD of patients 5 and 6 follow that of patient 4, but the low levels of VWF:Ag in their samples difficult the visualization of VWF multimers.

In the envenomed rat, the reduction in HMWM, VWF:Ag, VWF:CB, VWF:CB/VWF:Ag ratio, and platelet count suggested the presence of AVWS [17] in experimental *B. jararaca* envenomation. As shown in Fig. 2 (lanes 4 and 6), in the rat and mouse injected with *B. jararaca* venom, the decrease in the optical density of HMWM was followed by an increase in that of LMW multimers, indicating that systemic proteolysis of VWF was occurring during envenomation. Plasma ADAMTS13 levels from the envenomed rat (72.4 ng/mL) did not differ substantially from the control rat (89.1 ng/mL).

4. Discussion

VWF:MD analysis is essential for an adequate classification of VWD type and can provide valuable information to be considered for patient management. However, this technique has been dismissed in the diagnostic routine practice because, until now, it required a laborious procedure to achieve reliable results. Our technique provides a well-defined view of all different VWF multimer sizes.

Our main concern was to improve the technique and to speed up the procedure to facilitate its implementation, so that it can be used as a feasible diagnostic tool under clinical or research laboratory conditions. In fact, this technique is very flexible, and more electrophoresis chambers can be connected to a single power supply, so that more wells are available for VWF:MD analysis in a single day.

Wet transfer is preferred for larger proteins and it is less likely to dry out the gel [51]. Because of these features, wet transfer is the most commonly method used for VWF blotting. However, since semi-dry transfer has the advantage of blotting several gels simultaneously [52], we employed this type of protein transfer for analysis of VWF in agarose gels [32,53], so that non-specialized laboratories could analyze VWF:MD in a large number of samples in a short period of time.

Using sensitive methodologies for band detection like fluorescence, it is feasible to accomplish a discriminative quantitative VWF:MD analysis in 12 h. However, independently of the availability of fluorescence or chemiluminescence detecting equipment, the pattern of VWF:MD can also be observed using antibodies labeled with horseradish peroxidase or alkaline phosphatase, which require a substrate to develop a colored product. The chromogenic method is simple, achieves fast results, and does not require special equipment, but it cannot be used for quantitative analysis due to the poor contrast between the antibody reaction and the background [52].

Compared to previously reports [20,21,54], due to the relative short time needed to complete the assay, the laboratory staff may opt to analyze samples at once or can divide the task in two parts: in day one, to perform the electrophoresis and protein transfer, and in day two membrane development and band analysis. In the latter case, nitrocellulose membranes must be frozen at −20 °C immediately after they have dried in the incubator. Therefore, membranes can be stored and developed when needed.

The absence of HMWM and the increase in LMW multimers in rats and mice injected with *B. jararaca* venom suggest the occurrence of systemic VWF proteolysis and/or consumption. The presence of botrocetin in *B. jararaca* venom, which induces VWF consumption in vivo [55], or the activity of snake venom metalloproteinases (also belonging to ADAMs family) – which have been reported to degrade VWF unspecifically in distinct points [56,57] – might be accounted for this phenomenon. However, excessive amounts of plasmin engendered during *B. jararaca* envenomation [58], which have been reported to evoke AVWS [17], may also be involved in VWF proteolysis. We are currently investigating the pathophysiology of VWF alterations during experimental *B. jararaca* envenomation to understand it in more details. Thus, this technique is also suited for VWF:MD analysis in rats and mice, commonly used experimental models for thrombotic and hemorrhagic diseases. Besides, it can be used apparently to diagnose VWD in other animal species, such as dogs and pigs, which also suffer from VWD [51,54].

In conclusion, the optimization carried out herein on previous protocols shortened the time and facilitated the procedure for VWF:MD analysis in plasma samples. The whole process required 12 h, from gel preparation to membrane development. It is noteworthy that more electrophoresis chambers can be used, allowing a great number of samples to be analyzed simultaneously. Fluorophores are preferred for VWF:MD analysis, but if photo documentation systems are not available, DAB can be used for qualitative analysis. Thus, this technique provides a cheap, faster and easier method for VWF:MD analysis.

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Conflict of interest

The authors declare no financial/commercial conflict of interest.

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