Review Article

Guidelines on the laboratory diagnosis of congenital bleeding disorders in Pakistan

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Abstract

Congenital bleeding disorders are found in all racial groups and are present worldwide. Among all of them haemophilia A, B and Von Willebrand's disease are the commonest and they are characterized by the low blood levels of factor VIII, IX and Von Willebrand's factor respectively. Severity of bleeding is proportional to the severity of factor deficiency. The diagnosis of bleeding disorders can be complex, and no single diagnostic tests are suitable for all patients. The guideline was developed after reviewing relevant publications, summarizing current understanding of bleeding disorders and classification, and present a consensus diagnostic recommendation based on analysis of the literature and expert opinion. They also suggest an approach for clinical and laboratory evaluation of individuals with bleeding symptoms, history of bleeding or conditions associated with increased bleeding risk. The document summarizes needs for improvement in laboratory testing and quality which is very much needed in Pakistan to make a correct diagnosis, train master trainers, identify complications of bleeding disorders in local population, increase awareness among masses, involve government in haemophilia care, education of patients and their families and health care community. It further enhances the need for research in bleeding disorders, including clinical research to obtain more objective information about bleeding symptoms, advancements in diagnostic and therapeutic tools.

Keywords: Congenital bleeding disorders, Pakistan, Haemophilia A, Von Willebrand disease, Platelet functional disorder, Inhibitors.

Introduction

Haemostasis refers to the process whereby blood coagulation is initiated and terminated in a tightly regulated fashion, together with the removal of the clot as part of vascular remodelling. Thus haemostatic system is a complex pathway which integrates five major components; blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolytic elements. Whenever bleeding occurs the haemostatic plug is formed within few minutes of trauma and results from a complicated process involving vascular responses, platelet aggregation and activation of the blood coagulation mechanism.[1] Congenital bleeding disorders comprise a heterogeneous group of diseases which reflect abnormalities of blood vessels, coagulation proteins and platelets. Among all of them haemophilia A, von Willebrand's disease and haemophilia B are the commonest and they are characterized by the low blood levels of factor VIII, von Willebrand's factor and factor IX respectively. Therefore, these patients have a tendency to bleed; severity and type of bleeding is proportional to the severity of factor deficiency so that these patients are classified as mild, moderate and severe disease.[2] There are some other, comparatively uncommon bleeding disorders which results from poor platelet functions.

Haemophilia is the most common inherited bleeding disorder, resulting in lifelong crippling arthropathy. Haemophilia A and haemophilia B are X- linked recessive disorders affecting males of all ethnic groups; females are carriers. The number of affected persons worldwide is about 400,000.[3] Clinically haemophilia A is undistinguishable from haemophilia B so the laboratory diagnosis of haemophilia is essential to differentiate them. The incidence of haemophilia A (factor VIII deficiency) is 1 in 5000 male live births and that of haemophilia B (factor IX deficiency) is 1 in 30000.[1,2]

Von Willebrand Disease (VWD) is a relatively common bleeding disorder caused by deficiency or dysfunction of von Willebrand's factor. VWD is associated with a defect in primary haemostasis and also with a secondary defect in coagulation factor VIII. Congenital quantitative or qualitative defects of VWF are seen in VWD, which typically manifests as mucocutaneous bleeding, with nose bleeding, menorrhagia, and bleeding after tooth extraction as leading symptoms. Due to the high complexity of VWD, a whole panel of laboratory tests is required to diagnose and classify VWD, an essential step for the subsequent clinical management.[4] The true prevalence of VWD has not been clearly established, which may be due to variability of clinical and laboratory manifestations. There is limited information on VWD in the developing countries like Pakistan. The data showed that in most countries, the ratio of patients with VWD to severe haemophilia varied between 0.1 to 0.6 with a mean of approximately 0.4, as opposed to an

expected ratio of approximately 1 (based on population-based data from Italy), confirming the under diagnosis of VWD even within the same health-care system. Worldwide VWD prevalence is generally 1% of the normal population with higher frequency of type -1 VWD.[5,6]

Platelets play a key role in the primary haemostasis through aggregation at the injury site. The inherited platelet disorders are an uncommon cause of symptomatic bleeding. But relatively more common in communities where consanguineous marriages are more frequent like in the Middle East and India, high prevalence as compared to the West is reported in the literature.[7-9] Abnormalities of platelet function are characterized by clinical bleeding of varying severity. Congenital platelet disorders can alter circulating platelet numbers, function or both. These conditions are typically manifested by symptoms of excessive mucocutaneous bleeding, rapid onset, and excessive bleeding following invasive procedures or due to trauma.[10,11] However, there is considerable heterogeneity in the severity of bleeding problems associated with congenital platelet disorders, and in the defects that alter platelet function and or numbers.[12]

Methods of Literature Searches:

Three section outlines, approved by the Expert Panel chair, were used as the basis for compiling relevant search terms, using the Medical Subject Headings (MeSH terms) of the MEDLINE database. If appropriate terms were not available in MeSH, then relevant non-MeSH key words were used. In addition to the search terms, inclusion and exclusion criteria were defined on the basis of feedback from the Panel about specific limits to include in the search strategies, specifically the following:

1. date restriction: 1990-2009;

2. language: English; and

3. study/publication types: randomized-controlled trial; meta-analysis; controlled clinical trial; epidemiological studies; prospective studies; multicentre study; clinical trial; evaluation studies; practice guideline; review, academic; review, multicase; technical report; validation studies; review of reported cases; case reports; journal article (to exclude letters, editorials, news, etc.).

The search strategies were constructed and executed in the MEDLINE database to compile a set of citations and abstracts for each section. Initial searches on specific key word combinations and date and language limits were further refined by using the publication type limits to produce results that more closely matched the section outlines. Once the section results were compiled, the results were put in priority order by study type as follows:

1. Randomized-controlled trial;

2. Meta-analysis (quantitative summary combining results of independent studies);

3. Controlled clinical trial;

4. Multicentre study;

5. Clinical trial (includes all types and phases of clinical trials);

6. Evaluation studies;

7. Practice guideline (for specific healthcare guidelines);

8. Epidemiological studies;

9. Prospective studies;

10. Review, academic (comprehensive, critical, or analytical review);

11. Review, multicase (review with epidemiological applications);

12. Technical report;

13. Validation studies;

14. Review of reported cases (review of known cases of a disease); and

15. Case reports.

On examination of the yield of the initial literature search, it was determined that important areas in the section outlines were not addressed by the citations. As a result, the references used in the guidelines included those retrieved from the two literature searches combined with the references suggested by the Panel members. These references inform the guidelines and clinical recommendations, on the basis of the best available evidence in combination with the Panel's expertise and consensus.

Clinical recommendations — grading and levels of evidence:

Recommendations made in this document are based on the levels of evidence described in Table-1, with a priority grading system of A, B or C. Grade A is reserved for recommendations based on evidence levels Ia and Ib. Grade B is given for recommendations having evidence levels of IIa, IIb and III. Grade C is for recommendations based on evidence level IV. None of the recommendations merited a grade of A. Evidence tables are available on the NHLBI Web site for those recommendations graded as B with two or more references.

Approach to the diagnosis of bleeding disorder:

1. The patient history:

History taking is a key part of the assessment of a possible bleeding disorder and is the best screening method.

Table-1: Level of evidence.

Level	Type of evidence
Ia	Evidence obtained from meta-analysis of randomized-controlled trials
Ib	Evidence obtained from at least one randomized-controlled trial
IIa	Evidence obtained from at least one well-designed controlled study without randomization
IIb	Evidence obtained from at least one other type of well-designed quasi-experimental study
III	Evidence obtained from well-designed non-experimental descriptive studies, such as comparative studies, correlation studies and case–control studies
IV	Evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities.

This includes personal as well as family history of bleeding. Type of bleeding, onset, site, duration and associated clinical features. Has the patient needed medical attention for bleeding? During or after surgery? After dental work? With trauma? Is there any history of bruises or haematoma? (Grade B, level IIb).

A drug history should be taken, such as Aspirin, NSAIDs, oral anticoagulants and other drugs are the commonest cause of platelet dysfunction (Grade C, level IV).[13]

2. Bleeding manifestations:

◆ Are important as type of bleeding gives clue to underlying defects;

◆ unexplained or extensive bruising, purpura, ecchymoses;

◆ epistaxis particularly lasting for more than 30 min, causing anaemia or hospital admission seen in platelet disorders, VWD (Grade C, level IV).

◆ menorrhagia since menarche suggests VWD as well as in platelet disorders.

◆ Oral cavity bleeding.

◆ Bleeding during childbirth.

◆ Bleeding following dental extraction or invasive procedures.

◆ Post circumcision bleeding, haemarthrosis, haematomas are characteristic of coagulation disorders (Grade B, level IIb).[1,14]

Is there a past history of:

a. Liver or kidney disease?

b. A blood or bone marrow disorder?

c. A high or low platelet count? (Grade C, level IV).[13]

Laboratory diagnosis:

Screening tests provide an assessment of the extrinsic and intrinsic systems of blood coagulation system and also the conversion of fibrinogen to fibrin. These tests include:

Complete blood count and peripheral film examination:

Gives assessment of all three cell lines, particularly platelet count should be measured. A peripheral blood film examined to confirm the platelet count, size and morphology. Any changes in white blood cells or red cells should be noted. If clumping is seen, a platelet count should be repeated in a citrated anticoagulant.[15]

Coagulation Screen:

All patients for a suspected bleeding disorder should have a prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time and / or Clauss fibrinogen assay. Local normal reference ranges should be determined by the laboratories and use of sensitive reagents for the tests (Grade C, level IV).[14] The variability associated with coagulation assays makes the use of reliable reference materials essential. Performance of coagulation tests depends on proper handling of sampling and reagents. This includes pre-analytical, analytical, and post analytical variables.[15]

Pre-analytical variables:

Venous blood should be collected from the patient in a relaxed and calm environment. Whenever possible, sample should be collected without a pressure cuff, to minimize the effects of contact activation. The blood thoroughly mixed with the anticoagulant by inverting and collected up to the mark on the tube. Labelling the patient sample both at the bed side and within the laboratory is very important.

Blood sample anticoagulation; the most commonly used anticoagulant for coagulation samples is 0.109M (3.2%) trisodium citrate in a ratio of 9 parts blood to 1 part anticoagulant and fill volume must be at least 90% of the target volume for some test system to give accurate results. If the patient has a haematocrit greater than 55%, results of PT and APTT can be affected and the volume of anticoagulant should be adjusted to take account of the altered plasma volume. Immediate transportation of the sample to the laboratory is essential to prevent loss of the labile clotting factors.

Centrifugation should be performed without delay at 1200-1500 x G for 15 minutes for platelet poor plasma. The platelet count should be below 104/micro L. Centrifugation at a temperature of 18-25°C is acceptable for most clotting tests. After centrifugation, prolonged storage at 4-8°C should be avoided as this can cause cold activation, increasing FVII activity and shortening of the PT. Baseline coagulation tests Prothrombin time, Activated partial thromboplastin time and fibrinogen assay should be carried

out as soon as possible (within a 2-4 hours of blood collection) (Grade C, level IV).[15,16]

For platelet rich plasma (PRP) to investigate platelet function, samples should be centrifuged at room temperature (18-25°C) at 150-200g for 15 minutes, and analyzed within 2 hours of sample collection.

Samples can be saved in liquid nitrogen (-196°C) or may be frozen at -40°C or -80oC for several weeks without loss of most haemostatic activity. Once thawed at 37°C for 3-5 minutes, the sample should never be refrozen. If frozen samples are shipped to another laboratory for testing on dry ice, care must be taken to avoid exposure of the plasma to carbon dioxide which may affect the pH and the results of screening tests (Grade C, level IV).[15,17]

Analytical variables:

Calibration and Quality Control (QC):

To assess the precision of a particular method it is essential to perform repeated analysis of aliquots of the same sample. It is important to include QC samples with normal and abnormal values to ensure that a method is under control at different levels of a particular analyte. Since relatively minor changes in an analytical process may be more apparent when testing an abnormal control. Controls should be included with patient samples in a batch of tests. It must be of stable and homogenous material. However, fresh normal control sample is required for tests such as platelet aggregation. Whenever possible, the normal pool should be calibrated against a reference material. In the absence of reference material a large normal pool should be used and assigned a value of 100 u/dl. (Grade C, level IV).

Reagents and Equipments:

All reagents used in the tests should be prepared according to the manufacture's instructions, stored properly, date of expiry, opening and labelling maintained. Similarly all equipments used should be calibrated and laboratory safety measures should be followed. Validation of a new lot of reagents with the previous lot is also important. (Grade C, level IV).[15,16]

Post analytical:

The most common errors at this stage are clerical. These can be minimized by thorough scrutiny of the results before their authorization for printing.

When a test result is abnormal or unexpected, the results should be checked from previous test results, if available. The samples must be checked for clots and when possible the test should be repeated. It is always better to give an interpretation of the test results with a possible diagnosis and suggested further investigations. Timely reporting of results to the concerned person and dispatch of reports.[15] Critical results of INR (>5.0) and APTT (>65 seconds) should be promptly communicated to care givers or patients.

First line screening tests:

Prothrombin Time (PT):

The PT test measures the clotting time of plasma in the presence of an optimal concentration of tissue extract (thromboplastin) and indicates the overall efficiency of the extrinsic clotting system. The test is known to depend also on reactions with factors V, VII, X, II and fibrinogen concentration. It is particularly useful in monitoring anticoagulation in patients on warfarin. The sensitivity of the test is influenced by the reagent and technique used and it is important to establish a reference range locally. For oral anticoagulant monitoring, thromboplastin with an international sensitivity index (ISI) of 1.0 should be selected (Grade B, level IIb).[15,18]

Expression of Results:

The results are expressed as the mean of the duplicate readings in seconds or as the ratio of the mean patient's plasma time to the mean normal control plasma time.

PT of patient's plasma in seconds and PT of control plasma in seconds.

PT is considered abnormal if there is a difference of more than 3 sec between control and test results.[19]

PT is expressed in INR. INR = R^{ISI}

R = ratio between PT of patient and control plasma.

ISI = it is given with the package insert of the reagent. Duplicate clotting times should not differ by more than 10%. Normal values depend on the thromboplastin used, the exact technique, and whether visual or instrumental end-point reading is used.[16,17]

INR is required for monitoring patients on oral anticoagulant therapy.

Activated Partial Thromboplastin Time (APTT):

Abnormalities in the intrinsic and common pathway will result in prolongation of the APTT. Taken together with

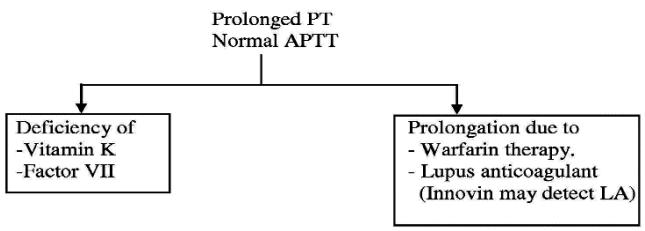

Figure-1: Workup of Prolonged PT.

a normal prothrombin time, it is the most useful screening test for detecting deficiencies of factors VIII, IX, XI and XII. The APTT will also be prolonged in any deficiency involving the common pathways (deficiencies of factors V, X, II and to a lesser extent fibrinogen) and in the presence of inhibitors. The presence of some therapeutic inhibitors of coagulation, such as heparin and lupus anticoagulant, will also prolong APTT.[19] Some of the reagents are particularly sensitive to detect deficiencies of clotting factors while others are sensitive to pick up lupus anticoagulants. Both problems will produce a prolonged APTT. Selection of reagent should be based on the nature of the hospital laboratory workload and the type of patients mainly tested. Testing mainly paediatric population, it is advisable to use an APTT reagent which is sensitive to detect a possible underlying clotting factor deficiency while testing adults would require a reagent which could detect a lupus anticoagulant. In a hospital setting where anticoagulation therapy monitoring is the main workload, heparin sensitive reagent is desirable. It is important to rule out these treatments (heparin) as a cause of prolonged APTT before continuing with other tests. (Grade C, level IV).

Expression of Results:

Express the results as the mean of the paired clotting times.

- APTT of the patient plasma in seconds.
- APTT of the control plasma in seconds.
- Result expressed in APTT ratio.

Normal Range:

A normal range should be established locally. The normal range is typically within 26-40 sec. The actual times depend on the reagents used and the duration of the pre incubation period, which varies in manufacturer's recommendations for different reagents.[15,16]

Prolonged APTT:

The difference between APTT of patient's plasma and that of control plasma is more than 6.0 seconds.[15]

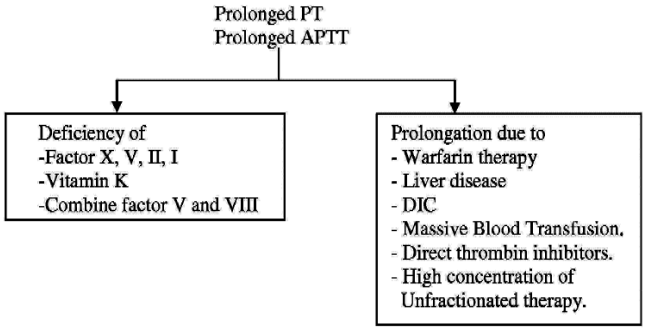

Figure-2: Workup of Prolonged PT and APTT.

Thrombin Time:

The thrombin time measures the rate of conversion of fibrinogen to polymerized fibrin after the addition of thrombin to plasma. It is sensitive to and thus prolonged in:

- Hypo-and dysfibrinogenaemia
- Heparin therapy
- The presence of fibrin(ogen) degradation products and factors that influence the fibrin polymerization (e.g. the presence of a paraprotein in Myeloma)

Expression of Results:

The results are expressed as the mean of the duplicate clotting times in seconds for the control and the test plasma. A patients TT Should be within 2 seconds of the control 15-19 second (Grade C, level IV).[15]

Fibrinogen Assay:

A number of methods are available for the measurement of fibrinogen concentration which includes clotting, immunological, physical and nephelometric techniques but all give slightly different results due to heterogeneous nature of plasma fibrinogen. The recommended method for measuring fibrinogen concentration as originally described by Clauss is based on the thrombin time and uses a high concentration of thrombin solution (Grade C, level IV).[15]

Normal Range:

The normal range is 2-4g/l.

If there is a strong bleeding history and yet screening tests are normal then tests should be repeated to exclude mild VWD and mild haemophilia.

Mixing Tests:

Abnormal screening tests, i.e. PT/APTT or both can be further investigated to determine the cause of the abnormality. Information on the nature of the defect can usually be obtained by mixing studies. The test plasma is mixed with normal plasma or plasma with a known coagulation defect. Correction indicates a possible factor deficiency, whereas failure to correct suggests the presence of an inhibitor (Grade C, level IV).[15,16]

Factor Assays:

The assays of factor VIII: C, IX, XI or XII are activated partial thromboplastin time based, were as factor II, V, VII and X based on the prothrombin time. Routinely one-stage assay is used. The assay compares the ability of dilutions of a standard or reference plasma and test plasma to correct the APTT/PT of plasma known to be totally deficient in the clotting factor being measured but which contains all

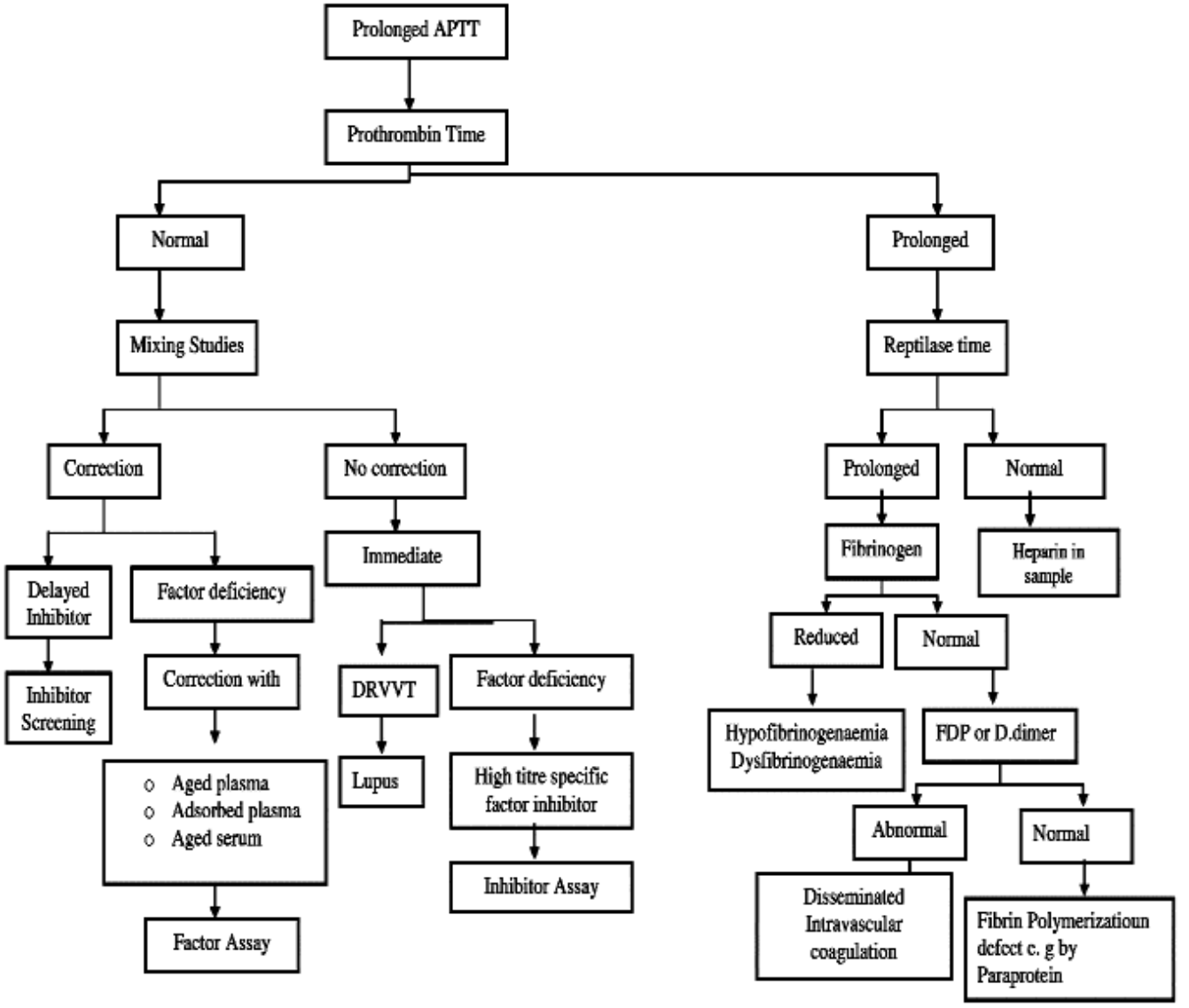

Figure-3: Workup of Prolonged APTT.

other factors required for normal clotting.[15,18]

Bleeding Time:

The utility of the test is limited because it is poorly reproducible, invasive, insensitive, and time consuming. It does not correlate with the bleeding tendency within individual patients and it is widely considered that an accurate bleeding history is a more valuable screening test. Nowadays in developed world many centres use Platelet function analyser-100 screening test for platelet disorders. But in developing part of the world and in Pakistan also still bleeding time is used widely as a screening tool. The advantage is that it is a simple test of natural haemostasis including the important contribution of the vessel wall and it also avoids potential anticoagulation. It is advisable to check the platelet count before carrying out the bleeding time test. Patients with a platelet count below 50 x 10^9/L may have a very long bleeding time and the bleeding may be difficult to arrest. A number of different methods have been described but as it is subject to a large number of variables and confounding factors, it needs standardization of the method. However, a normal bleeding time does not mean normal haemostasis therefore good history and clinical correlation is important (Grade C, level IV).[15]

Platelet Aggregation:

Platelet aggregation done with platelet-rich plasma gives important information about platelet function. Different

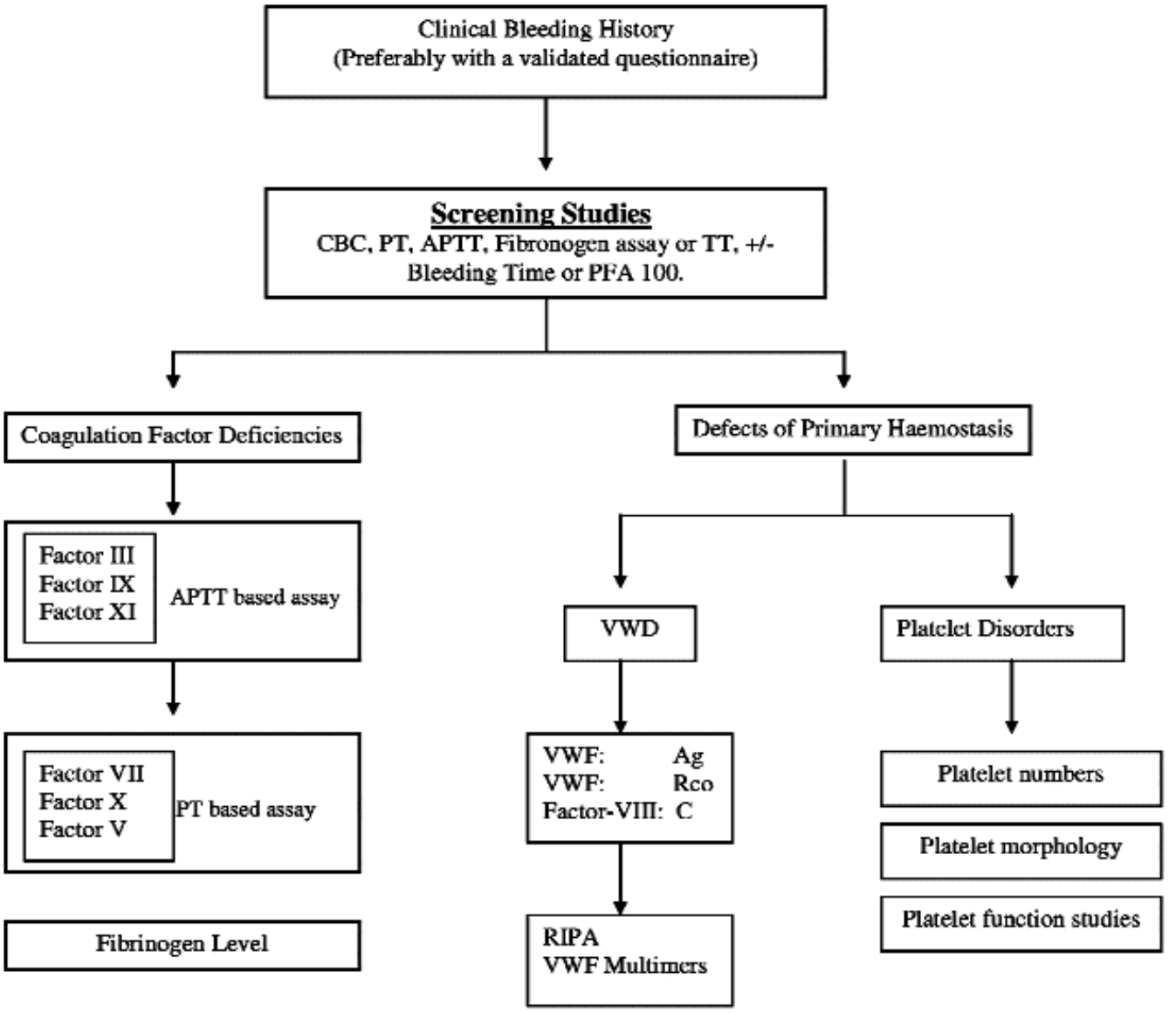

Figure-4: Diagnostic laboratory workout in patients with bleeding disorders.

agonists used are ADP, adrenaline, collagen, arachidonic acid, ristocetin, and thrombin. Control from normal healthy donors is used in conjunction with the patient sample and compared.[15] In thrombocytopenic samples, where platelet count is below 120 x 10^9/L, the best options are to adjust a control sample to the same count as the patient or to perform studies on washed platelets where the platelet number can be normalized. However, neither case is ideal therefore more specialist tests should be used such as flow cytometry (Grade C recommendation based on level IV evidence).[20,21]

Inhibitors:

Haemophilia A and B are X linked recessive bleeding disorders caused by the deficiency of factor VIII and factor IX respectively. The main stay of treatment is replacement of the deficient clotting factor. About 20 to 30% of haemophilia A patients develop inhibitors. They are more common in severe type as compared to mild to moderate types. Studies showed that large deletions, stop codon mutations and inversion 22 are associated with greater chances of production of inhibitor as compared to small deletions and missense mutations (35% vs. 5%) and a positive family history of inhibitor formation . FVIII and FIX mutation analysis should be done in all patients with haemophilia A and B, especially newly diagnosed patients (grade B, level IIb). However, molecular diagnostics of bleeding disorders

are largely unavailable in Pakistan due to lack of expertise and facilities. Recently, inversion of intron 22 has been started in Lahore haemophilia centre for antenatal screening in severe haemophilia. The inhibitor development in haemophilia B is relatively uncommon occurring in 2 to 3% patients. These antibodies are exclusively IgG isotype. These inhibitors neutralize the procoagulant activity of factor by blocking functional epitopes as well as they are in a capacity to hydrolyse the antigen for which they are specific. The presence of inhibitors have significant clinical implications as the response to treatment becomes uncertain, morbidity is increased and life expectancy reduced. Inhibitors develop in patients with severe haemophilia A after a median of 9-12 treatment days but may arise at any time in the patient's life.[24] Inhibitor development is less common in patients who have received more than 150 exposure days of factor concentrate replacement.[25] However; factor IX inhibitors may be associated with life threatening anaphylactic reactions.[26]

Laboratory assessment in inhibitor screening:

The screening for inhibitors will be dependent on the treatment regimen used by the patient. Patients should be screened for inhibitors after every 5th exposure day or every 3 months until the 20th Exposure day, every 6-12 months thereafter and prior to any surgical or invasive procedure. Patients should also be screened for inhibitors if the frequency of bleeding increases or if the clinical or laboratory response to replacement therapy is poor (Grade C, level IV).[27]

Inhibitor screening using the activated partial thromboplastin time (APTT) or Bethesda based method:

APTT based screening method for detection of factor VIII inhibitor, which is time and temperature dependent. Normal plasma mixed with plasma containing an immediately acting inhibitor will have little or no effect on the prolonged clotting time. In contrast, if normal plasma is added to plasma containing a time-dependant inhibitor, the clotting time of the latter will be substantially shortened. However, after 1-2 hours, correction will be abolished, and the clotting time will become long again. To detect both types of inhibition, normal plasma and test plasma samples are tested immediately after mixing and also after incubation together at 37°C for 120 min. Each laboratory should standardize the test and establish ranges (Grade C, level IV).[15,27]

Factor VIII inhibitor quantification:

The Bethesda assay is the recommended standard method for measuring the factor VIII inhibitor titre. However, the original Bethesda method may give false positive at low levels (below 1.0) which can be eliminated by utilizing Nijmegen modification which by stabilizing pH and protein concentrations and therefore eliminates loss of factor VIII activity. For these, 0.1M imidazole at pH 7.4 and immunodepleted factor VIII deficient plasma are added in the control mixture (Grade C, level IV).[28]

A Bethesda unit is defined as the amount of inhibitors which will neutralize 50% of one unit of added factor VIII in 2 hours at 37°C.[16]

Factor IX Inhibitors:

Factor IX inhibitors can be quantified using the Bethesda method, but as factor IX inhibitors act immediately, 5 to 15 minute's incubation is needed depending upon the procedure used. There is no published literature on the role of the Nijmegen modification for the factor IX inhibitors (Grade C, level IV).[15,26]

Factor VIII/ IX recovery:

Factor VIII/IX recovery is calculated from subtracting the pre-infusion level from the post infusion and reported as adjusted in vivo recovery (IVR) on a per dosage basis as IU/dl or IU/dl per IU/kg. A pre-infusion sample should be taken and a post infusion sample taken 15-30 minutes after the end of the infusion. The dose /kg are calculated by patient's weight.

Normal adjusted IVR for plasma derived FVIII for older children and adults are between 2.0 and 2.5 IU/dl/IU/kg can vary slightly according to product. Recombinant FVIII has similar recovery values. FIX IVR values are lower than FVIII because factor IX has a much larger volume of distribution. FIX IVR values range from 0.7 -1.7.[29] Data for both FVIII and FIX are spared for infants less than 1 year old. (Grade C, level IV).

Factor VIII/ FIX half -life studies:

Half life studies are conducted after a wash out period of at least 72 hours or when the base line factor level is reached (typically <1.0 IU /dl). To obtain maximum information it is recommended that the following samples are taken at; 15 minutes, 30 minutes, 3 hours, 6 hours, 9 hours, and 24 hours, and with additional samples at 28 and 32 hours for FVIII and 48, 72 hours for FIX.[30] The mean half life (T1/2) for plasma derived FVIII in adults range between 10 and 15 hours. Factor IX concentrates have a much longer T1/2 than FVIII ranging from 7 to 34 hours (Grade C, level IV).[31]

Laboratory Diagnosis of Von Willebrand disease (VWD):

VWD is classified into three major categories: partial quantitative deficiency (type 1), qualitative deficiency (type 2), and total deficiency (type 3). Type 2 VWD is divided further into four variants (2A, 2B, 2M, 2N) on the basis of

details of the phenotype. Evaluation based on personal or family history of bleeding, particularly mucocutaneous type, or female with menorrhagia though not a specific marker of VWD.[4] Studies have reported a prevalence of VWD of between 5% and 20% among women who have menorrhagia.[32,33] An initial haemostasis laboratory work up includes a bleeding time, platelet count and complete blood count (CBC), activated partial thromboplastin time (APTT), prothrombin time (PT), and optionally either a fibrinogen level or a thrombin time (TT).This testing neither "rules in" nor "rules out" VWD, but can suggest whether coagulation factor deficiency or thrombocytopenia might be the cause of clinical bleeding. The initial tests commonly offered in initial VWD diagnosis are only available in few teaching hospitals of larger cities of Pakistan. These three tests, VWF: Ag is an immunoassay which measures the amount of VWF protein present in plasma. The function of the VWF protein that is present as ristocetin cofactor activity (VWF: RiCoF) measures the ability of VWF interaction with normal platelets. The ability of the VWF to serve as the carrier protein to maintain normal FVIII level in the body is measured by FVIII: C. In Pakistan and in developing world the assay is usually performed as a one-stage clotting assay based on the APTT. The clotting assay, is done using an automated or semi automated instrument, measures the ability of plasma FVIII to shorten the clotting time of FVIII-deficient plasma. As this test is important in the diagnosis of haemophilia, the efforts to standardize this assay have been greater than for other haemostasis assays. FVIII activity is labile, with the potential for spuriously low assay results if blood specimen collection, transport, or processing is suboptimal. If any of the tests are abnormal further tests should be performed/ repeating the test or referred to a specialized centre (Grade B, level III).[6,34]

The VWF: RiCoF to VWF: Ag ratio:

Mainly in Pakistan this ratio assists in the diagnosis of types 2A, 2B, and 2M VWD and help to differentiate them from type 1 VWD. A VWF: RiCoF / VWF: Ag ratio <0.7 has been used as a criterion for dysfunctional VWF (type-2).[6] Similarly another method has been proposed for the use of the VWF: CB/VWF: Ag ratio. In type 2A VWD, the ratio is usually low; and in type 2B VWD, the VWF: RiCoF/VWF: Ag ratio is usually low but may be normal. In type 2M VWD, the VWF: Ag concentration may be reduced or normal, but the VWF: RiCoF/VWF: Ag ratio will be <0.7.[35]

ABO blood groups:

The blood groups have a significant effect on plasma VWF: Ag. Individuals who have blood group O have concentrations approximately 25 percent lower compared to persons who have other ABO blood groups. Therefore, it has been recommended to establish local reference ranges for VWF: Ag and VWF: RiCoF with respect to blood groups.[6,36]

Other assays for Sub Classification of VWD:

VWF multimer analysis is a qualitative assay that illustrates the variable concentrations of the different-sized VWF multimers by using sodium dodecyl sulphate (SDS)-protein electrophoresis followed by detection of the VWF multimers in the gel, using a radio labelled polyclonal antibody/or a combination of monoclonal antibodies. It helps to differentiate type 2 VWD subtypes from type 1 and type 3 and is performed after the initial VWD testing. VWD multimer assay and other additional tests like VWF: platelet binding, VWF collagen-binding, and VWF: FVIII binding required for sub classification and diagnosis are not available at present in Pakistan and need to be developed.[32,33]

DNA sequencing analysis:

DNA sequencing of patient DNA has been used to make a molecular diagnosis of variants of type 2 VWD,[6,35] but DNA sequencing is not available in our country. Due to lack of expertise, research facilities, and awareness about the disease among the health care community (Grade C, level IV). Preliminary data on molecular genetics from our neighbouring country suggests that there are significant population differences.[36]

Special consideration for Laboratory Diagnosis of VWD:

Repeated testing for VWD is sometimes needed to identify low levels of VWF. Stress — including surgery, exercise, anxiety, crying in a frightened child, as well as systemic inflammation, pregnancy, or use of estrogen/oral contraceptives — can cause an increase in plasma levels of VWF and mask lower baseline values.[6] Therefore, patients with suggestive history and borderline levels, the tests should be repeated particularly in females during menstruation, when factor levels are at their lowest.

Disclosures:

The authors state that they have no interest which might be perceived as posing a conflict or bias.

Table-1: Collection and Handling of Plasma Samples for Laboratory Testing.[6]

Phlebotomy conditions — An a traumatic blood draw limits the exposure of tissue factor from the site and the activation of clotting factors, minimizing falsely high or low values.

Patient stress level — Undue stress, such as struggling or crying in children or anxiety in adults, may falsely elevate vWF and FVIII levels. Very recent exercise

can also elevate vWF levels.

Additional conditions in the person — The presence of an acute or chronic inflammatory illness may elevate vWF and FVIII levels, as may pregnancy or administration of estrogen/oral contraceptives.

Sample processing — To prevent cryoprecipitation of VWF and other proteins, blood samples for VWF assays should be transported to the laboratory at room temperature. Plasma should be separated from blood cells promptly at room temperature, and the plasma should be centrifuged thoroughly to remove platelets. If plasma samples will be assayed within 2 hours, they should be kept at room temperature. Frozen plasma samples should be carefully thawed at 37°C and kept at room temperature for <2 hours before assay.

Sample storage — Plasma samples that will be stored or transported to a reference laboratory must be frozen promptly at or below -40°C and remain frozen until assayed. A control sample that is drawn, processed, stored, and transported under the same conditions as the tested person's sample may be helpful in indicating problems in the handling of important test samples.

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