3 LABORATORY DIAGNOSIS

1. A correct diagnosis is essential to ensure that a patient gets the appropriate treatment. Different bleeding disorders may have very similar symptoms.

2. Accurate diagnosis can only be made with the support of a comprehensive and accurate laboratory service. This is dependent on the laboratory following strict protocols and procedures, which require:

   - knowledge and expertise in coagulation laboratory testing
   - use of the correct equipment and reagents
   - quality assurance

3. For detailed information on technical aspects and specific instructions on screening tests and factor assays, please consult the WFH's *Diagnosis of Hemophilia and Other Bleeding Disorders: A Laboratory Manual, Second edition* [1].

3.1 Knowledge and expertise in coagulation laboratory testing

Principles of diagnosis

1. Understanding the clinical features of hemophilia and the appropriateness of the clinical diagnosis.

2. Using screening tests to identify the potential cause of bleeding, for example, platelet count, bleeding time (BT; in select situations), or other platelet function screening tests, prothrombin time (PT), and activated partial thromboplastin time (APTT).

3. Confirmation of diagnosis by factor assays and other appropriate specific investigations.

Technical aspects

Preparation of the patient prior to taking a blood sample

1. Fasting is not normally necessary before collection of blood for investigation of possible bleeding disorders, although a gross excess of lipids may affect some automated analysers.

2. Patients should avoid medications that can affect test results such as aspirin, which can severely affect platelet function and prolong the bleeding/closure time.

3. Patients should avoid strenuous exercise immediately prior to venipuncture.

4. If a patient is particularly stressed by the sample collection procedure, the levels of FVIII and von Willebrand factor may be temporarily elevated.

Sample collection

1. The sample should be collected as per standard guidelines [2].

2. The sample should preferably be collected near the laboratory to ensure quick transport.

3. Samples should be tested within four hours of collection.

4. Results of tests can change according to the sample storage conditions. Higher temperatures (>25°C) lead to loss of FVIII activity over time, whereas sample storage in the cold (2-8°C) leads to cold activation. The sample should therefore be maintained at temperatures between 20°C and 25°C where possible, but for no more than four hours.

5. Venipuncture must be clean and the sample collected within one minute of tourniquet application without prolonged venous stasis.

6. Blood should be withdrawn into a plastic syringe or an evacuated collection system. The needle should be 19-21 gauge for adults and 22-23 gauge for small children. Collection through peripheral venous catheters or non-heparinized central venous catheters can be successful for many tests of hemostasis.

7. Blood from an indwelling catheter should be avoided for coagulation tests.

8. Frothing of the blood sample should also be avoided. It is often useful to discard the first 2 ml of blood collected.

9. The sample should be collected in citrate tubes containing 0.105M–0.109M (c3.2%) aqueous trisodium citrate dihydrate, maintaining the proportion of blood to citrate as 9:1. If the tube contains less than 80% of the target volume, results may be adversely affected. The higher strength concentration of 3.8% trisodium citrate is no longer recommended.

10. Prompt and adequate mixing with citrate solution should be done by gentle inversion.

11. If the sample cannot be processed within four hours of collection, the platelet poor plasma can be frozen at -30°C and stored for a few weeks, or up to six months if stored at -70°C [3]. Storage at -20°C is usually inadequate.

12. Frozen samples must be thawed rapidly for four to five minutes at 37°C to avoid formation of cryoprecipitate.

Preparation of platelet-poor plasma (PPP)

1. PPP should be prepared as per standard guidelines [2].

2. PPP is prepared by centrifugation of a sample at a minimum of 1700g for at least 10 minutes at room temperature (i.e. not refrigerated).

3. PPP may be kept at room temperature (20°C–25°C) prior to testing.

4. Plasma that has been hemolysed during collection and processing should not be analysed.

End-point detection

1. Many laboratories now have some form of semi or fully automated coagulation analysers. Accurately detecting the clotting end-point using a manual technique requires considerable expertise, particularly if the clotting time is prolonged or if the fibrinogen concentration is low, and the clot is thin and wispy.

2. For manual testing, the tube should be tilted three times every five seconds through an angle of approximately 90° during observation. The tube should be immersed in a water bath at 37°C between tilting.

Screening tests

1. Platelet count, BT, PT, and APTT may be used to screen a patient suspected of having a bleeding disorder [4].

2. Bleeding time lacks sensitivity and specificity and is also prone to performance-related errors. Therefore other tests of platelet function such as platelet aggregometry are preferred when available [5,6].

TABLE 3-1: INTERPRETATION OF SCREENING TESTS

POSSIBLE DIAGNOSIS	PT	APTT*	BT	PLATELET COUNT
Normal	Normal	Normal	Normal	Normal
Hemophilia A or B**	Normal	Prolonged*	Normal	Normal
VWD	Normal	Normal or prolonged*	Normal or prolonged	Normal or reduced
Platelet defect	Normal	Normal	Normal or prolonged	Normal or reduced

* Results of APTT measurements are highly dependent on the laboratory method used for analysis.
** The same pattern can occur in the presence of FXI, FXII, prekallikrein, or high molecular weight kininogen deficiencies.

3. Based on the results of these tests, the category of bleeding disorder may be partially characterized to guide subsequent analysis (see Table 3-1, above).

4. These screening tests may not detect abnormalities in patients with mild bleeding disorders including some defects of platelet function, FXIII deficiency, and those rare defects of fibrinolysis, which may be associated with a bleeding tendency.

Correction studies

1. Correction or mixing studies using pooled normal plasma (PNP) will help to define whether prolonged coagulation times are due to factor deficiency or circulating anticoagulants of inhibitors. Correction studies with FVIII/FIX-deficient plasma may be used to identify the particular deficiency if a factor assay is not available.

Factor assays

1. Factor assay is required in the following situations:
   - To determine diagnosis
   - To monitor treatment
     - The laboratory monitoring of clotting factor concentrates is possible by measuring pre- and post-infusion clotting factor levels.
     - Lower than expected recovery and/or reduced half-life of infused clotting factor may be an early indicator of the presence of inhibitors.
   - To test the quality of cryoprecipitate
     - It is useful to check the FVIII concentration present in cryoprecipitate as part of the quality control of this product.

2. Phenotypic tests lack sensitivity and specificity for the detection of carriers. Some obligate carriers may have a normal FVIII:C/VWF:Ag ratio. Genotypic testing is a more precise method of carrier detection and is therefore recommended.

3. One-stage assays based on APTT are the most commonly used techniques. The following assay features are important:
   - FVIII- and FIX-deficient plasma must completely lack FVIII and FIX respectively, i.e. contain < 1 IU/dl, and have normal levels of other clotting factors.
   - The reference/calibration plasma, whether commercial or locally prepared, must be calibrated in international units (i.e. against an appropriate WHO international standard).
   - At least three different dilutions of the reference plasma and the test sample under analysis are needed for a valid assay.
   - Use of a single dilution of test sample substantially reduces the precision of the test and may lead to completely inaccurate results in the presence of some inhibitors.
   - When assaying test samples from subjects with moderate or severe hemophilia, an extended or separate calibration curve may be needed. It is not acceptable to simply extend the calibration curve by extrapolation without analysing additional dilutions of the calibration plasma.
   - Some cases of genetically confirmed mild hemophilia A have normal FVIII activity when the one-stage assay is used for diagnosis, but reduced activity in chromogenic and two-stage clotting assays. The reverse can also occur. This means that more than one type of FVIII assay is needed to detect all forms of mild hemophilia A [7,8].

Inhibitor testing

1. The presence of some form of inhibitor is suspected when there is a prolonged APTT that is not fully corrected by mixing patient plasma with PNP.

2. The most frequently encountered functional inhibitors of hemostasis are lupus anticoagulants (LA), which are not directed against specific clotting factors and which should be excluded.

3. Results of APTT testing on mixtures of test and normal plasma can be difficult to interpret, particularly since in acquired hemophilia there may initially be a full correction of APTT in the presence of a potent specific anti-FVIII antibody.

4. Most FVIII inhibitors that occur secondary to replacement therapy in subjects with hemophilia A show a characteristic pattern: the APTT of a patient/PNP mixture is intermediate, i.e. between the APTTs of the two materials, and is further prolonged when the mixture is incubated at 37°C for 1-2 hours.

5. Confirmation that an inhibitor is directed against a specific clotting factor requires a specific inhibitor assay.

6. **The Nijmegen modification of the FVIII inhibitor assay offers improved specificity and sensitivity over the original Bethesda assay. (Level 1) [9,10]**

7. It is performed as follows:

- Buffered PNP (providing FVIII) is mixed with test plasma and incubated at 37°C.
- After two hours, the residual FVIII is measured by comparison against the FVIII in a control mixture comprised of buffered PNP and FVIII-deficient plasma, which has been incubated alongside the test mixture.
- Residual FVIII is converted into inhibitor units using a semi-log plot of the residual FVIII against inhibitor convention, which has been constructed using the assumption that 100% residual = 0 BU/ml inhibitor, and 50% residual = 1.0 BU/ml (the latter being the internationally agreed convention for defining inhibitor activity).
- When residual FVIII activity is <25%, the patient plasma must be retested after dilution to avoid underestimation of the inhibitor potency.
- An inhibitor titer of ≥ 0.6 BU/ml is to be taken as clinically significant [11].

Trained personnel

1. Even the simplest coagulation screening tests are complex by nature.

2. A laboratory scientist/technologist with an interest in coagulation must have an in-depth understanding of the tests in order to achieve accurate results.

3. In some cases, it may be beneficial to have a laboratory scientist/technologist who has had further training in a specialist centre.

3.2   Use of the correct equipment and reagents

1. Equipment and reagents are the tools of the trade of any laboratory. The following requirements are necessary for accurate laboratory testing.

Equipment

1. A 37°C ± 0.5°C water bath.

2. A good light source placed near the water bath to accurately observe clot formation.

3. Stopwatches.

4. Automated pipettes (either fixed or variable volume) capable of delivering 0.1 ml and 0.2 ml accurately and precisely.

5. Clean soda glass test tubes (7.5 cm × 1.2 cm) for clotting tests. Reuse of any glassware consumables should be avoided whenever possible, unless it can be demonstrated that test results are unaffected by the process used. Plasticware used in coagulation analysers should not be re-used.

6. An increasingly large number of semi-automated and fully automated coagulometers are now available. In many cases this equipment has the following advantages:
   - Accuracy of end-point reading.
   - Improved precision of test results.
   - Ability to perform multiple clot-based assays.
   - Reduction of observation errors (the end-point of the reaction is typically measured electro-mechanically or photoelectrically).
   - Use of polystyrene (clear) cuvettes instead of glass tubes.

7. All equipment requires maintenance to be kept in good working order.
   - When equipment is purchased consideration should be given to, and resources put aside for, regular maintenance by a product specialist.
   - Pipettes should be checked for accurate sample/reagent delivery.
   - Water baths, refrigerators, and freezers should undergo regular temperature checks.

8. Good results can be obtained using basic equipment and technology provided that good laboratory practice is observed. These skills can then be adapted to more automated technology.

Selection of coagulometers

1. Many coagulation analysers are provided as a package of instrument and reagent, and both components can influence the results obtained. This needs to be taken into account when evaluating and selecting a system. Other important issues to consider are:
   - type of tests to be performed and the workload, as well as workflow, in the laboratory
   - operational requirements (power, space, humidity, temperature, etc)
   - service requirements and breakdown response
   - throughput and test repertoire
   - costs
   - ability to combine with reagents from other manufacturers
   - user-programmable testing
   - comparability between results on primary analyser and any back-up methods
   - compatibility with blood sample tubes and plasma storage containers in local use

   - safety assessment (mechanical, electrical, microbiological)
   - availability of suitable training

2. Information is required in relation to the performance characteristics of the system. This can be obtained from a variety of sources including the published literature and manufacturers' data, but may also require some form of local assessment. Aspects to consider include:
   - precision of testing with a target of <3% of CV for screening tests and <5% for factor assays
   - carry-over
   - interfering substances
   - reagent stability on board analyser
   - comparability with other methods
   - sample identification
   - data handling, software, and quality control
   - training required
   - reliability

3. A number of published guidelines and recommendations describe the evaluation of coagulation analysers [12,13].

Reagents

1. It is good practice to ensure continuity of supply of a chosen reagent, with attention paid to continuity of batches and long shelf-life. This may be achieved by asking the supplier to batch hold for the laboratory, if possible.

2. Changing to a different source of material is not recommended unless there are supply problems or because of questionable results. Different brands may have completely different sensitivities and should not be run side by side.

3. Instructions supplied with the reagent should be followed.

4. Particular attention should be paid to reagent stability. Once a reagent is reconstituted or thawed for daily use, there is potential for deterioration over time depending on the conditions of storage and use.

5. Once an appropriate test and reagents have been decided upon, normal/reference ranges should ideally be defined, and must take account of the conditions used locally.

3.3   Quality assurance

1. Quality assurance (QA) is an umbrella term used to describe all measures taken to ensure the reliability of laboratory testing and reporting.

2. QA covers all aspects of the diagnosis process from sample-taking, separation and analysis, and internal quality control through to reporting of the result and ensuring that it reaches the clinician.

3. It is the responsibility of everyone involved to make sure that the procedures are followed in the correct manner.

Internal quality control (IQC)

1. IQC is used to establish whether a series of techniques and procedures is being performed consistently over a period of time.

2. IQC measures are taken to ensure that the results of laboratory investigations are reliable enough to assist clinical decision making, monitor therapy, and diagnose hemostatic abnormalities.

3. IQC is particularly useful to identify the degree of precision of a particular technique.

4. For screening tests of hemostasis, normal and abnormal plasma samples should be included regularly. At least one level of IQC sample should be included with all batches of tests.

External quality assessment (EQA)

1. Laboratories are strongly advised to participate in an external quality assessment scheme (EQAS) to audit the effectiveness of the IQC systems in place.

2. EQAS helps to identify the degree of agreement between the laboratory results and those obtained by other laboratories.

3. Participation in such a scheme helps build confidence between a laboratory and its users.

4. The WFH IEQAS is specifically designed to meet the needs of hemophilia treatment centres worldwide. The scheme includes analyses relevant to the diagnosis and management of bleeding. Details of this scheme, which is operated in conjunction with the U.K. National External Quality Assessment Service for Blood Coagulation in Sheffield, U.K., can be obtained from the WFH [14].

5. Other national and international quality assessment schemes are also available.

6. In order for a laboratory to attain a high level of testing reliability and to participate successfully in EQAS, it must have access to appropriate reagents and techniques and an appropriate number of adequately trained staff.

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