Role of the laboratory in ITI

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Basic equipment

- centrifuge (generating at least 1500 g, better 2500 g)
- coagulometer (fully automated, semi-automated)
  - optical
  - mechanical
- regulated water bath (capable of maintaining temperatures of 37 ± 0.5 °C)
- refrigerator for reagent storage (+2 to +8 °C)
- deep freezer (able to maintain at least -25 °C, better -70 °C)
- calibrated automatic pipettes
- stopwatch(es)

Specialized staff (lab technicians)

Coagulation laboratory

Sample collection

- 0.109M (3.2%) or 0.129M (3.8%) sodium citrate anticoagulant (1 part of anticoagulant : 9 parts of blood)

- Sample volume – nominal value ± 10% (NCCLS)

- When sampling, use the „second tube“ for coagulation!

- First circa 2 ml of drawn blood should not be used for tests of haemostasis
Sample collection

- **Haematocrit > 0.60 and < 0.25** — the volume of anticoagulant is specifically calculated from the formula:

\[ V_{\text{citrate}} = \frac{100 - \text{haematocrit(\%)} \times 3.81}{595 - \text{haematocrit(\%)} \times 3.81} \text{ml blood} \]

Transport of the sample

- Up to 2 hours at room temperature +15 to +25 °C for whole blood samples
- Frozen plasma samples at -20 °C and below can be stored/transported much longer

Pre-analytical variables

- **Centrifugation**
  - PPP (platelet-poor-plasma) - at minimum 1500 g for at least 10 min at room temperature +15 to +25 °C
  - Cold activates FVII and system of kallikreins
  - Refrigerated centrifuge (< +15 °C) — specific samples only (e.g. anti-Xa, PAI...)
  - Some test procedures require the plasma to be centrifuged twice (e.g. LA)

- **Testing of the sample**
  - Samples should be tested within 4 hours of sample collection when possible (it depends on the test procedure — e.g. aPTT within 2 hours, samples with heparin within 1 hour)
Pre-analytical variables

- **Deep-frozen plasma samples**
  - Better to freeze plasma at -196 °C ("shock-freezing" in liquid nitrogen)
  - Storage at -70 °C or lower is preferred (some clotting factors can be then tested even after 6 months of storage)
  - Storage at -20 °C (max 1 month)
  - Thawing for 5-10 min at +37 °C, then mix by reversing the tube gently

Quality Control

- **Internal quality control (IQC)**
  - Intermediate precision (results ± 2 SD, mean, CV,...)
  - Reproducibility
  - Commercial QC with target range of acceptable values
  - Quality control between instruments

- **External quality assessment (EQA)**
  - Is used to identify the degree of agreement between one laboratory’s results and those obtained by other centres

- **ISO (certification, accreditation of the laboratory)**
Normal reference range

- Respect age, sex, ABO group (F VIII, vWF)
- Follow national/international recommendation
- Check the literature
- Respect manufacturer’s information/recommendation
- You may also rely on your own laboratory reference range (healthy normal subjects - reference range should include the central 95% of values)

Activated partial thromboplastin time (aPTT)

- Screening aPTT reagent:
  - sensitive to defect of factors
  - sensitive to heparin (UHF)
  - sensitive to LA

- aPTT reagent for determination of factors, inhibitors:
  - non-sensitive to LA
  - sensitive to defects of factors

Carefully select your aPTT reagent!

aPTT

- Calibration
  - aPTT time for ‘normal plasma’ in seconds (mean of repeated measurements)
    - calibrator = ‘normal plasma’
    - (pooled normal plasma (PNP) or commercially provided normal plasma)

- Results
  - time (seconds)
  - Ratio (R)
  \[ R = \frac{t_{\text{pac}}}{t_{\text{norm}}} \]
### aPTT

**Normal reference range**

- **Age**
  - 0 – 1 m: 0,8 – 1,5
  - 1 m – 1 y: 0,8 – 1,3
  - > 1 y: 0,8 – 1,2

  - Time in seconds is significantly dependent on reagent and instrument used!

**Clinical relevance**

- Hypo coagulation (prolonged coagulation time)
- Hyper coagulation is not relevantly mirrored in aPTT!
  - shortened aPTT times – low sensitivity – no clinical relevance (except sampling errors)

### aPTT

**Prolonged aPTT:**

- Defect of factors: VIII, IX, XI, XII, PK, HMWK
- Defect of factors V, X, II (the common pathway, PT longer too)
- VWD
- Dys- or afibrinogenemia
- Presence of inhibitors (specific, non-specific)
- Presence of heparin (UFH)
- Presence of FDP
- Physiologically – newborns (R = 0,8 – 1,6)
  - children up to age 1 year (R = 0,8 – 1,3)
- Sampling errors

### Correction (mixture) tests

- For further investigation of abnormal aPTT (when PT is normal) or PT (when aPTT is normal)

- N (normal plasma)
- P (patient’s plasma)
- M (mix = N+P (1+1))

- Determination of aPTT/PT
Correction (mixture) tests

- Is the defect in patient’s plasma corrected adding normal plasma?
  - YES → aPTT/PT correction of more than 50% → factor deficiency is very likely
  - Poor correction or no correction → inhibitor to one of the clotting factors (specific inhibitor) or non-specific inhibitor (e.g., LA) → circulating anticoagulant

Circulating anticoagulant

- Identification of time-dependent / time-independent inhibitor
- Specific inhibitor (against coagulation factor)
- Assays based on aPTT/PT

- Tubes: N, P, 4N+1P, 1N+1P, 1N+4P
- Determination of aPTT/PT
- Incubation for 2 hours at 37°C
- Determination of aPTT/PT
Circulating anticoagulant

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<th>time-dependent inhibitor</th>
<th>immediate-acting inhibitor</th>
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<td>normal plasma</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>patient’s plasma</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>P+P (1:1) before incubation</td>
<td>41</td>
<td>45</td>
<td>70</td>
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Time (s)
Incubation for 2 hours at +37 ° C

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Circulating anticoagulant

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Circulating anticoagulant

- **Calculate:**
  - \(4N+1P\): \(4 \times \text{time (N)} + 1 \times \text{time (P)} / 5\)
  - \(1P+1N\): \(1 \times \text{time (P)} + 1 \times \text{time (N)} / 2\)
  - \(1N+4P\): \(1 \times \text{time (N)} + 4 \times \text{time (P)} / 5\)

- **Identification of Inhibitor:**
  - Addition of 1/5 patient’s plasma to 4/5 of normal plasma causes markedly prolonged time of normal plasma sample.
F VIII (one-stage assay)

- Activity of the F VIII is determined by modified aPTT test using correction of aPTT in FVIII-deficient plasma by FVIII present in patient’s plasma

- FVIII-deficient plasma
  - available commercially
  - very low or undetectable level of the FVIII (FVIII < 1%)
  - high activity of all other factors
  - no anti-FVIII antibodies

F VIII (one-stage assay)

- Measurement of the FVIII:
  - 1 part of diluted patient’s plasma (1:10 with buffer OVB)
  - 1 part of FVIII deficient plasma
  - 1 part of aPTT reagent, incubation
  - 1 part of CaCl₂

- Determination of aPTT (in seconds)
- Derive % FVIII from the calibration curve

F VIII – calibration

- Calibrator (calibrated against WHO standard, when possible) with defined factor activity
- The calibration curve (min. 4 dilutions) – double logarithmic plot
F VIII:C

- Note!
  - Not all combinations of deficiency plasma and aPTT reagent work equally well!

- Significant role
  - Quality of the deficient plasma
  - The sensitivity of the aPTT reagent to factor deficiency

- F IX
  - The same procedure but with F IX deficient plasma

F VIII:C – test for low levels

- FVIII < 10% (with standard dilution of the sample 1:10)
  - Use lower dilution 1:5 (or 1:2,…) depending on the instrument and the calibration curve used

- FVIII low – calibration curve used (e.g. < 10%)

F VIII:C – test for low levels

- FVIII low - calibration curve (e.g. < 10%)
  - Dilution of the standard plasma with the FVIII deficient plasma (recommended for optical coagulometers)

- Quality control
  - Commercial lyophilized human plasmas with defined factor activity (2 levels: N and P)
Normal reference range

<table>
<thead>
<tr>
<th>Age</th>
<th>FVIII (%)</th>
<th>FIX (%)</th>
</tr>
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<tbody>
<tr>
<td>&lt; 1d</td>
<td>60 – 140</td>
<td>20 – 75</td>
</tr>
<tr>
<td>1d – 1m</td>
<td>60 – 125</td>
<td>65 – 110</td>
</tr>
<tr>
<td>1m – 1y</td>
<td>55 – 100</td>
<td>50 – 125</td>
</tr>
<tr>
<td>1y – 6y</td>
<td>75 – 150</td>
<td>50 – 110</td>
</tr>
<tr>
<td>6y – 18y</td>
<td>50 – 150</td>
<td>60 – 150</td>
</tr>
<tr>
<td>&gt; 18y</td>
<td>50 – 150</td>
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(Recommendation of the Czech Haematology Society, 2013)

Defects of F VIII

- Congenital
  - Hemophilia A

- Acquired
  - Due to acquired inhibitors (antibodies)

Chromogenic F VIII assay

- More than 20% of mild hemophilia A patients show discrepancy between activity of F VIII determined by one-stage assay and chromogenic assay

- Patients with normal aPTT, normal F VIII (one-stage assay) but with personal or family history of mild hemophilia → use chromogenic FVIII assay
Quantitative measurement of FVIII inhibitors

- Time-dependent inhibitors
- Show linear inhibition kinetics (type I)
- The presence of an inhibitor might be suspected from a reduced half-life and recovery of FVIII
- Bethesda (BA), Nijmegen, Oxford assay

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Quantitative measurement of FVIII inhibitors

One Bethesda unit (BU) is defined as the amount of an inhibitor that will neutralize 50% of FVIII present in normal plasma after 2 hours of incubation at +37 °C.

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Quantitative measurement of FVIII inhibitors (BA)

- Different dilutions of the plasma sample with imidazole buffer (1:2, 1:4, 1:8, 1:16, ..., 1:256)
  - in case of strong inhibitor, use more titres (1:512, 1:1024, ...)
- FVIII inhibitor positive controls (QC)
- Control mixture (imidazole buffer)
- Mix the sample with the equal part of normal plasma (pooled plasma)
- Incubation for 2 hours at +37 °C
- Inhibitors inactivate FVIII present in the normal plasma
Quantitative measurement of FVIII inhibitors (BA)

- After incubation at +37 °C the activity of the FVIII in patients’ samples and control mixtures is determined.

- Calculate residual activity of the factor (%):
  \[
  \text{residual activity} = \frac{\text{activity of the factor in the sample}}{\text{activity of the factor in control mixture}} \times 100
  \]

- Calculate separately for patient’s samples and positive controls.

- Dilution of the patient’s plasma that gives a residual FVIII nearest to 50 % but within the range 30 - 60 % (event. 25 – 70 %) is chosen for calculation of the inhibitor.

The inhibitor activity is calculated from a graph of residual FVIII activity versus inhibitor units (BU).

Derive the inhibitor titre from the graph and multiply by the dilution to give the final titre.

Quantitative measurement of FVIII inhibitors

- Normal values:
  - < 0.6 BU/ml

- Inhibitor:
  - < 5 BU/ml Low responder
  - > 5 BU/ml High responder

Inhibitor: < 5 BU/ml Low responder
> 5 BU/ml High responder
The Nijmegen modification

- Use buffering the normal plasma with 0.1M imidazole buffer at pH 7.4
- Use immunodepleted FVIII deficient plasma in the control mixture and for dilution of the patient samples

**Advantage:**
- Is better at low inhibitor titres (<1 BU) than classical Bethesda assay (Bethesda assay can result in false positivity)
- ISTH recommendation (at least for CCC)

The Oxford modification

- Use a concentrate of the FVIII instead of normal plasma
- Use 4 hours incubation at +37 °C
- Not often used these days...

Quantitative measurement of FVIII inhibitors

- **Potential problems (troubleshooting)**
  - Determination of the inhibitor during IT treatment – interference of the residual FVIII in the sample – false low titre of the inhibitor or negativity of the inhibitor
- **Possible resolution**
  - Heat the test plasma at +58 °C for 90 min (will destroy all the clotting factors including FVIII, but not the inhibitor – it is heat resistant)
### FVIII inhibitors

- Type of the inhibitors (type I and II) depends on the kinetics of the inhibitor.

![Graph showing Type I and Type II FVIII inhibitors](image)

### Recovery and half-life

- The sampling points:
  - 0 (baseline), 0.25, 0.5, 1, 3, 6, 9, 12, 24, 32, 48, 72 h
  - at least 5 time-points: patient ≤ 6 years old

- ISTH recommendation

### Recovery

- Ratio of desired and real increase of FVIII/FIX (IU/dl) in patient’s plasma after the dose of injected factor (in IU/kg)
  - Given in percentage
  - 1 IU/kg of FVIII should increase the plasma level in 2 %, for FIX in 1 %
  - Is often calculated from the highest measured FVIII/FIX plasma concentration of FVIII/FIX within the first hour post infusion
  - Recovery shall be more than 66 % (hemophilia A)
Recovery and half-life

• Half-life
  – The period of time required for the concentration or amount of drug in the body to be reduced to exactly one-half of a given concentration or amount
  – Half-life greater than 7 hours (hemophilia A)

Thank you for your attention.
Questions welcome! ☺

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