Laboratory Investigation of Challenging Cases

Laura A. Worfolk, Ph.D
Scientific Director, Coagulation
Disclosures

- Employee of Quest Diagnostics

- Case focus:
  - Importance of knowing test limitation/interpretations
  - Differentiation of inhibitors
Cascade and Laboratory Testing

Intrinsic & Contact Pathway

- XIIa
- Xla
- IXa/VIIa

Extrinsic Pathway

- VIIa/TF

Common Pathway

- DRVVT
- aPTT
- PT

Hexagonal Phase

Prothrombin

Thrombin

Fibrinogen → Fibrin Clot

Injury

Confidential – Do not copy or distribute
Case #1

- 52-year-old female: PTT mixing study ordered (PT normal)
- Possibilities:
  - Factor deficiency (VIII, IX, XI, XII)
  - Lupus Anticoagulant (prolongs aPTT, generally not PT)
  - Von Willebrand Disease (not likely)
  - Pre-analytic factor (but PT is normal & prolongation is reproducible)
  - Heparin (prolongs aPTT, but not PT)
Abnormal aPTT corrected after mixing with normal pool plasma, suggesting factor deficiency....
Case #1: Factor Testing

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII</td>
<td>66%</td>
<td>50-180%</td>
</tr>
<tr>
<td>Factor IX</td>
<td>151%</td>
<td>60-160%</td>
</tr>
<tr>
<td>Factor XI</td>
<td>126%</td>
<td>65-150%</td>
</tr>
<tr>
<td>Factor XII</td>
<td>136%</td>
<td>50-150%</td>
</tr>
</tbody>
</table>

Inhibitor pattern was not detected with any of the factor assays.....
Inhibitors and Factor Testing

• Multiple dilutions of patient sample are tested to ensure an inhibitor is not affecting results; examples below:

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>Normal</th>
<th>Heparin</th>
<th>FVIII Inhibitor</th>
<th>Lupus Anticoagulant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>105%</td>
<td>15%</td>
<td>&lt;1%</td>
<td>85%</td>
</tr>
<tr>
<td>1:20</td>
<td>99%</td>
<td>45%</td>
<td>&lt;1%</td>
<td>127%</td>
</tr>
<tr>
<td>1:40</td>
<td>108%</td>
<td>63%</td>
<td>&lt;1%</td>
<td>154%</td>
</tr>
<tr>
<td>1:80</td>
<td>104%</td>
<td>??</td>
<td>??</td>
<td>??</td>
</tr>
<tr>
<td>Final</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Case #1 Continued: Additional Testing

- Von Willebrand Factor testing all normal
- Lupus Anticoagulant testing negative
- What else?

- Prekallikrein: 1% (60-187%); asymptomatic, prolonged PTT picked up during pre-op screening

\[\text{XII} \rightarrow \text{XIIa} \]
\[\text{XI} \rightarrow \text{XIIa} \]
\[\text{HMWK/Prekallikrein} \]
\[\text{IX} \rightarrow \text{IXa} \]
\[\text{VIII} \]
Prekallikrein (Fletcher Factor) Deficiency

- PK: 609 aa protein w/ 58% homology to FXI
  - 75% circulates bound to HK
- Deficiency first identified by Hathaway and colleagues in 1965, autosomal-recessive trait*
- Deficiency is not associated with a bleeding disorder, nor does it appear to protect against thrombotic disease
- Extremely rare (<100 case reports in the literature) and does not require treatment

*Blood 1965;26:521
Contact Pathway and the APTT Test

HK-PK → α-Kallikrein → FXII → FXIIa → FXI → FXIa

FXIIa is generated by contact activation and

APTT Contact Activators: Silica, Ellagic Acid, Kaolin

FXIIa → Thrombin Generation +CaCl2

Clot formation

HK=High Molecular Weight Kininogen
Contact Pathway and the APTTT Test

HK-PK → α-Kallikrein

FXII  APTTT Activator

FXIIa

Prekallikrein

APTT Contact Activators: Silica, Ellagic Acid, Kaolin

FXI

FXIa

+CaCl2  Thrombin Generation

Prolonged Clotting Time

HK=High Molecular Weight Kininogen
PK Deficiency and Modified APTT

<table>
<thead>
<tr>
<th></th>
<th>APTT Incubation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>5 min</td>
</tr>
<tr>
<td>Normal Control</td>
<td>26.3 sec</td>
</tr>
<tr>
<td>Abnormal Control</td>
<td>40.5 sec</td>
</tr>
<tr>
<td>Patient</td>
<td>50.4 sec</td>
</tr>
</tbody>
</table>

**Patient PK level was 15%**

Prekallikrein Biologic Function

• Fibrinolysis
  – Alpha-kallikrein activates Pro-uropkinase, releasing urokinase

• Complement activation

• Kinin generation

Thrombosis & Hemostasis. 2007:5:1106-12
Case #2

• 79-year-old female: sample received for Lupus Anticoagulant workup

• Lupus Anticoagulants (LA):
  – One type of antiphospholipid antibody (APA) chx by prolongation of in-vitro phospholipid dependent clotting reactions
  – LA’s more closely associated with thrombotic events than other APAs
How to Test for LAs?

• Demonstration of phospholipid dependence using 2 different methods

• Evidence of inhibition by mixing studies

• Blood collection: before initiating or after discontinuing anticoagulant therapy; *platelet poor*

• LA can not be conclusively determined if the TT is significantly prolonged

• *Rule out specific factor inhibitors*

How Do We Test?

**Pathway #1**

PTT-LA

- **Prolonged**
  - Hexagonal Phase Confirm:
    - Pos or Neg

- **Normal**
  - Stop

*Pathway 1 or 2 positive: LA Detected*

**Pathway #2**

DRVVT Screen

- **Prolonged**
  - DRVVT Confirm
    - **Positive**
      - DRVVT Mix Study
      - Prolonged
      - LA Pos
      - Normal
  - **Negative**
    - Stop
  - **Normal**
    - Stop

- **Normal**
  - Stop
## LA Test Interpretation

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>PTT-LA</th>
<th>Hexagonal Phase</th>
<th>DRVVT Screen</th>
<th>DRVVT Confirm</th>
<th>DRVVT Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>Not Indicated</td>
<td>Normal</td>
<td>Not indicated</td>
<td></td>
</tr>
<tr>
<td>LA Positive</td>
<td>High</td>
<td>Positive</td>
<td>High</td>
<td>Positive</td>
<td>High (not corrected)</td>
</tr>
</tbody>
</table>
# LA Test Interpretation

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>PTT-LA</th>
<th>Hexagonal Phase</th>
<th>DRVVT Screen</th>
<th>DRVVT Confirm</th>
<th>DRVVT Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>Not Indicated</td>
<td>Normal</td>
<td>Not indicated</td>
<td></td>
</tr>
<tr>
<td>LA Positive</td>
<td>High</td>
<td>Positive</td>
<td>High</td>
<td>Positive</td>
<td>High (not corrected)</td>
</tr>
<tr>
<td>FVIII Inhibitor</td>
<td>High</td>
<td>Normal or Positive</td>
<td>Normal</td>
<td>Not Indicated</td>
<td></td>
</tr>
</tbody>
</table>
# LA Test Interpretation

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>PTT-LA</th>
<th>Hexagonal Phase</th>
<th>DRVVT Screen</th>
<th>DRVVT Confirm</th>
<th>DRVVT Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>High</td>
<td>Negative</td>
<td>Normal</td>
<td>Not Indicated</td>
<td></td>
</tr>
<tr>
<td>Coumadin</td>
<td>Normal-High</td>
<td>Negative</td>
<td>High</td>
<td>Normal-Pos</td>
<td>Normal (corrected)</td>
</tr>
</tbody>
</table>
Case #2 Continued: LA Work-up

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRVVT Screen (&lt;45 s)</td>
<td>36 s</td>
<td>PTT-LA (&lt;40 s)</td>
<td>95 s</td>
</tr>
<tr>
<td>DRVVT Confirm &amp; Mix</td>
<td>NI</td>
<td>Hexagonal Phase Confirm</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Overall interpretation: LA detected by one pathway...a bleeding history requires that other coagulopathies be excluded.....

<table>
<thead>
<tr>
<th>Result</th>
<th>Clinical History</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No past personal/family history of bleeding, inpatient for minor procedure but currently had large bruises on torso, no current or past thrombotic history....</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Result</th>
<th>Factor VIII Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>
Testing Caveats: FVIII vs LA

• Factor VIII inhibitors: may yield false positive Hexagonal Phase Confirm (& no effect on DRVVT)

• Lupus Anticoagulants: may falsely decrease clotting-based Factor Assays

• Besides clinical history, how to differentiate?
FVIII Inhibitors vs Lupus Anticoagulant

- Chromogenic Factor VIII assay not affected by lupus anticoagulants

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Clotting FVIII</th>
<th>Chromogenic FVIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>LA sample</td>
<td>30%/55%/86%</td>
<td>90%</td>
</tr>
<tr>
<td>FVIII Inhibitor</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>
**Test** | **Result**
---|---
FVIII Activity Clotting | <1%
FVIII Activity Chromogenic | <1%
FVIII Inhibitor | 461 Bethesda Units (BU) (Normal: <0.4 BU)

**Conclusion:** *FVIII inhibitor detected. Hexagonal Phase Confirm false positive due to FVIII inhibitor (DRVVT negative)*
Acquired Factor VIII Inhibitors

- Autoantibodies in non-hemophiliac patients are rare:
  - 1-4/million/year
  - high rate of morbidity/mortality
  - Incidence increases with age; biphasic age distribution: 20-30 years (post-partum population) & major peak 68-80 years
  - ~50% of cases: occur in patients lacking any disease

Franchini M, Lippi G. Blood. 2008;112:250-255
## Conditions Associated with Acquired Hemophilia A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automimmune disorders</td>
<td>SLE, rheumatoid arthritis, multiple sclerosis, Sjögren syndrome, autoimmune hemolytic anemia</td>
</tr>
<tr>
<td>Solid cancers</td>
<td>Prostate, lung, colon, pancreas, stomach, breast</td>
</tr>
<tr>
<td>Hematologic malignancies</td>
<td>CLL, non-Hodgkin lymphoma, multiple myeloma, Waldenström macroglobulinemia, myelodysplastic syndrome</td>
</tr>
<tr>
<td>Drug-associated</td>
<td>Penicillin &amp; its derivatives, sulfa antibiotics, phenytoin, chloramphenicol, methyldopa, clopidogrel</td>
</tr>
</tbody>
</table>

Adapted from: Franchini M, Lippi G. *Blood*. 2008;112:250-255
Diagnosis of Acquired Hemophilia A

• Laboratory Findings:
  – Isolated prolonged PTT which can’t be corrected by incubated mixing study
  – Reduced FVIII level
  – FVIII inhibitor activity detected by Bethesda or Nijmegen modification

• Clinically:
  – Hemorrhage into skin, muscles, soft tissues, mucous membranes; disease may manifest following trauma, surgery or by cerebral hemorrhage
Case #2 Continued: 7 months later….

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF Antigen</td>
<td>324%</td>
</tr>
<tr>
<td>FIX Activity</td>
<td>62% - 95% (inhibitor pattern)</td>
</tr>
<tr>
<td>FXI Activity</td>
<td>57% - 86% (inhibitor pattern)</td>
</tr>
<tr>
<td>Lupus anticoagulant &amp; Antiphospholipid Antibodies</td>
<td>DRVVT negative; <strong>Hexagonal positive</strong> Cardiolipin &amp; Beta-2-glycoprotein I negative</td>
</tr>
<tr>
<td><strong>FVIII Activity</strong></td>
<td>11% (possible inhibitor pattern, values off-curve)</td>
</tr>
<tr>
<td>Chromogenic VIII</td>
<td>7%</td>
</tr>
<tr>
<td><strong>FVIII Inhibitor</strong></td>
<td>50.6 BU</td>
</tr>
</tbody>
</table>
Case #3

- 59-year-old female: sample submitted for PT/PTT mixing study

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT &amp; PT Mix</td>
<td>80 sec / 48 sec, no correction</td>
</tr>
<tr>
<td>aPTT &amp; aPTT Mix</td>
<td>Vmax / 184 sec, no correction</td>
</tr>
<tr>
<td>Thrombin Time</td>
<td>18 s (16-23 sec)</td>
</tr>
</tbody>
</table>
## Case #3: Factor Testing

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>FII</th>
<th>FV</th>
<th>FX</th>
<th>FIX</th>
<th>FVIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>16%</td>
<td>&lt;1%</td>
<td>12%</td>
<td>Vmax</td>
<td>90%</td>
</tr>
<tr>
<td>1:20</td>
<td>62%</td>
<td></td>
<td>40%</td>
<td></td>
<td>173%</td>
</tr>
<tr>
<td>1:40</td>
<td>94%</td>
<td></td>
<td>99%</td>
<td></td>
<td>248%</td>
</tr>
</tbody>
</table>

Differential diagnosis: *Factor V Inhibitor or a monoclonal gammopathy*....
Case #3: Possibilities

• FV Inhibitors: extremely rare, <150 cases reported:
  – ~50% reported (1955-1997) developed in response to bovine FV (found in thrombin products, such as fibrin sealants applied to wound sites)
  – Major surgery is an independent risk factor, apart from use of bovine thrombin
  – β-lactam antibiotics
  – In ~20% of cases of anti-FV formation, no underlying disease identified

Case #3: Possibilities

• Monoclonal gammopathies: Multiple myeloma, Waldenstron’s macroglobulinemia, or primary amyloidosis or other lymphoproliferative disorders
  – M protein interference with coag testing
  – Rarely: FV inhibitor develops

Case #3: Inhibitor Testing

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V</td>
<td>23 BU / 25 BU</td>
</tr>
<tr>
<td>FII / X</td>
<td>With dilution, the factor activity is near normal, but can’t be precisely quantified. Since factor activity is near normal, the inhibitor assay not performed as a specific inhibitor would almost certainly not be detected.</td>
</tr>
</tbody>
</table>
Case #3: Conclusion

• Acquired Factor V Inhibitor interfering with PT- and PTT-based assays
  – Most patients will have a bleeding diathesis
  – Relationship to inhibitor titer and bleeding has not been studied
  – Some patients do not bleed: possible differential recognition of plasma & platelet Factor V; or antibody specificity (i.e. interference of APC inactivation of FVa)
Case #4

- 68-year-old female; high PT/PTT, multiple factors & LA tests ordered

- Possibilities:
  - Common pathway factor deficiency or multiple factor deficiencies
  - Thrombin inhibitor (i.e. Pradaxa)
  - Non-specific circulating inhibitor (i.e. monoclonal protein)
  - Pre-analytic factor (i.e. underfilled tube)
  - Lupus anticoagulant (unlikely, unless high-titer LA)
### Case #4: Continued

<table>
<thead>
<tr>
<th>Sample Dilution</th>
<th>FII/FV/FVII</th>
<th>FVIII</th>
<th>FXI</th>
<th>FXII</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5</td>
<td>&lt;5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>&lt;10%</td>
<td>4%</td>
<td>6%</td>
<td>8%</td>
</tr>
<tr>
<td>1:20</td>
<td>&lt;20%</td>
<td>45%</td>
<td>17%</td>
<td>21%</td>
</tr>
<tr>
<td>1:40</td>
<td>82%</td>
<td>38%</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td></td>
<td>70%</td>
<td>56%</td>
<td></td>
</tr>
</tbody>
</table>

PT = 73 sec, aPTT = 84 sec, Positive DRVVT
### Case #4: Continued

<table>
<thead>
<tr>
<th>Test</th>
<th>Result (Reference Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Willebrand Factor Antigen</td>
<td>363% (50-217%)</td>
</tr>
<tr>
<td>Ristocetin Cofactor Activity</td>
<td>473% (42-200%)</td>
</tr>
<tr>
<td>Cardiolipin IgM Antibody</td>
<td>&gt;100 U/mL (&lt;10 U/mL)</td>
</tr>
<tr>
<td>Cardiolipin IgA Antibody</td>
<td>31 U/mL (&lt;10 U/mL)</td>
</tr>
<tr>
<td>Cardiolipin IgG Antibody</td>
<td>&lt;10 U/mL (&lt;10 U/mL)</td>
</tr>
</tbody>
</table>
Case #4: Conclusion

- PT based factors (II, VII, X) undetectable, inhibitor patterns on multiple PTT based factors (VIII, XI, XII)
- DRVVT (a common pathway based test), strongly positive, Cardiolipin IgM strongly positive
- Elevated VWF antigen & activity

- Case conclusion: **IgM monoclonal gammopathy**
  - Monoclonal paraprotein interfering with PT/PTT & factor assays, true LA positive??
  - Suggest chromogenic FVIII, FX assays
Case #5

- 70-year-old woman with recurring massive nose bleeds. Her family doctor became concerned when the CBC showed a hemoglobin level of 5.5 g/dL
- Multiple tubes received for factor assays & fibrinogen
- “Lipemic” was handwritten on all tubes
## Case #5: Testing

<table>
<thead>
<tr>
<th>Test</th>
<th>Result/Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors VIII, IX, XI, &amp; XII</td>
<td>Sample appears to ‘clot’ in the instrument. All factors &gt;600%.</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>600 mg/dL</td>
</tr>
</tbody>
</table>

*Not valid results!*
Case #5: Observation

- Sample “clotted” on the analyzer, but would go back into solution with reheating

- Not a lipemic sample after all, but a cryoprecipitate instead
Case #5: Conclusion

- Suggested screen for multiple myeloma, which was positive

- Multiple myeloma patients may have:
  - Cryofibrinogenemia: antibodies to fibrinogen complex that precipitate in the cold (also interferes w/ fibrin polymerization)
  - Inability to polymerize fibrin: bleeding
Hemostatic Abnormalities and Multiple Myeloma

- MM associated with high risk of thrombosis
- Increased levels of VWF/FVIII with active disease, irrespective of treatment
- Increased levels of inflammatory cytokines (IL-6, TNF, CRP)
- Production of procoagulant auto-antibodies, i.e. lupus anticoagulant

Conclusions

• Knowledge of test limitations and clinical history yields the most cost-effective and clinically useful laboratory investigations

• Avoid test interpretation in isolation

• Use clinical history to guide test selection:
  – Diagnostic algorithms
  – Coagulation consults

Care360
from Quest Diagnostics
### Case #6: “A Rose by any Other Name”

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT, PTT, TT</td>
<td>Vmax</td>
<td><em>Is this a serum sample or Heparin contamination?</em></td>
</tr>
<tr>
<td>DRVVT Screen</td>
<td>Vmax</td>
<td></td>
</tr>
<tr>
<td>Hexagonal Phase Confirm</td>
<td>Negative</td>
<td><em>Negative for LA</em></td>
</tr>
<tr>
<td>Heparin Level</td>
<td>Less than 0.1</td>
<td><em>Negative for UFH</em></td>
</tr>
</tbody>
</table>

**Calcium = 18.2**
Case #6: Riddle Solved

- Sample labeled as plasma was really URINE!
  - BUN 1040
  - Total protein 11.0
  - Ca++ 18.2
  - *Smell = urine*

Manneken Pis (located in Brussels), Flemish for “Little Man Pee”
Case #6 Summary

• Why clot endpoint with the Hexagonal Phase Confirm?
  – Sample is mixed 1:1 w/ normal pool plasma in test system

• How did we prove it was urine?
  – BUN/Creatinine & calcium levels
  – “Whiff” test