Inhibitor Testing: State of the Art

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Overview

Background

Inhibitor Testing Methods

Hemophilia Inhibitor Research Study (HIRS)

National Inhibitor Surveillance (Community Counts)
What Are Inhibitors?

- Inhibitors are antibodies.
  - Our bodies make antibodies to fight diseases.
  - Antibodies are produced when the immune system does not recognize a protein entering the body.
  - Antibodies are part of the natural process to destroy a foreign substance.

- Inhibitors occur when the body does not recognize the normal clotting factor used for treatment.
  - Either the person with hemophilia (PWH) does not make any, or it is different from normal factor.

- Inhibitors act by combining with the factor and either blocking its action in clotting or removing it from the blood.
Risk Factors for Inhibitor Development

- Genetic:
  - Factor deficiency – FVIII>FIX
  - Severity of disease
  - Hemophilia gene defect
  - Family history of inhibitor
  - Race/ethnicity – Black and Hispanic rates two-fold higher than White
  - Immune response and modifying genes

- Treatment-related:
  - Frequency and intensity of exposure to factor products
  - Events surrounding treatment episodes
  - Type and structure of product used
Significance of Inhibitors

- Inhibitors often require a change in treatment.
  - Some inhibitors, called transient, disappear on their own.
  - Others may require the use of more factor but are not progressive.
  - The most significant inhibitors require:
    - use of a by-passing agent to produce clotting or
    - a process called immune tolerance induction to try to eliminate the inhibitor by giving frequent doses of clotting factor.

- Costs associated with inhibitors are staggering.
  - PWH with inhibitors are twice as likely to be hospitalized.
  - Cost of hospital care is 2-10 times greater.
  - Treating an inhibitor can cost up to $500,000 per year.

- Odds of death are 70% higher in inhibitor patients.
  - Analysis by Walsh et al of data collected by CDC Universal Data Collection (UDC) program
  - 42% of deaths in inhibitor patients were from bleeding, compared to 12% of deaths in non-inhibitor patients.
Inhibitor Testing Issues

- Proportion of people with severe hemophilia receiving an inhibitor test ranged across US centers from 0-100%, averaging 46% (UDC data).
- Inhibitors detected early are more easily treated by immune tolerance induction.
- Limitations to doing routine inhibitor testing in the U.S. (expert panel)
  - Requirement for “wash out” for testing
  - Lack of available laboratory expertise
  - Payment issues
  - High rate of false positive tests
- False positive rate is as high as 32%. The CV among laboratories is near 50%. (Favaloro et al. 2014, *Haemophilia* 20: Suppl 4)
Inhibitor Testing Issues

- Accurate inhibitor measurement is important for:
  - Clinical care
  - Evaluation of product safety
  - Assessment of population trends

- Clinically, inhibitor diagnosis is based on:
  - Laboratory findings
  - Response to therapy
  - Pharmacokinetic studies

- For surveillance and clinical trials, tests must be:
  - Accurate and reproducible
  - Usable during treatment
  - Confirmed, to minimize false positive results
Patient plasma

Pooled Normal plasma

Test reaction

Control reaction

Incubate @ 37°

Measure clot formation

Comparison of the amount of factor VIII or IX in the test reaction with the control reaction reflects the strength of the inhibitor in the patient sample.
History of Inhibitor Measurement

- **1959**: First report (Biggs and Bidwell)
  - Two-stage assay for Factor VIII

- **1975**: Bethesda Assay (Kasper et al)
  - One-stage assay for Factor VIII
  - Two hour incubation with pooled normal plasma
  - Established “Bethesda unit” for measurement

- **1995**: Nijmegen Assay (Verbruggen et al)
  - Modified Bethesda Assay using buffered pooled normal plasma and dilution with factor VIII-deficient plasma
  - More sensitive and specific
  - “Gold standard” for inhibitor testing

- **2012**: North American Specialized Coagulation Laboratory Association (NASCOLA) Survey (Pruthi et al)
  - 20% using the Nijmegen assay
  - 10% using the classical Bethesda assay
  - 70% using buffered normal plasma (hybrid)
Factor VIII Inhibitor Measurement Methods

Functional Assays

Antibody Detection Assays
**Factor VIII Inhibitor Measurement Methods**

**Functional Assays**

- **Nijmegen-Bethesda Assay (NBA)**
  - Measures inhibition of clot formation
  - Limitations:
    - Non-specific endpoint (fibrin clot)
    - Plasma components from multiple individuals
    - Influenced by LA, heparin, non-specific inhibitors
    - Insensitive (low-titer modification)
    - In vitro kinetics poorly understood

- **Chromogenic Bethesda Assay (CBA)**
  - Measures inhibition of FXa generation
  - Not influenced by LA, heparin, non-specific inhibitors
  - More specific for FVIII inhibitors
Factor VIII Inhibitor Measurement Methods

Antibody Detection Assays

- Detect both inhibitory and non-inhibitory antibodies
- Not primary assays: require follow-up with functional assays for confirmation and quantitation
- More sensitive than functional assays
- May be used to confirm FVIII reactivity
- Enzyme linked immunosorbent assay (ELISA)
  - Antibody binding to FVIII immobilized on a plastic surface
  - Commercially available
- Fluorescence-based immunoassay (FLI)
  - Antibody binding to FVIII immobilized on fluorescent beads
Fluorescence-based immunoassay for detecting anti-Factor VIII antibodies in plasma

1) biotinylated anti-human Ig
2) PE-streptavidin

Carboxylated polystyrene bead

Kogenate coupled polystyrene bead

incubate beads with plasma samples
Hemophilia Inhibitor Research Study (HIRS)

- Study conducted by the CDC at 17 US hemophilia treatment centers, beginning in 2006, funded by the CDC Foundation with grants from Pfizer and Baxter
- Prospective treatment data collected on 1163 patients from 17 sites
  - Followed for 3329 person-years
  - Records for 113,205 exposure days
- 23 new inhibitors detected: 9 at enrollment, 14 during follow up
- Population at risk for inhibitors includes all patients:
  - One-third of new inhibitors were in non-severe patients.
  - One-half were over age 5.
  - One quarter had >150 exposure days.
  - 61% had no clinical effect at detection.

HIRS Inhibitor Testing

- 2590 specimens for FVIII and 567 for FIX inhibitor testing

- Laboratory goals:
  - To adapt NBA for high-throughput testing
  - To establish quality control
  - To determine cut-off for a positive inhibitor
  - To evaluate alternative methods

- Key Findings:
  - Developed and validated a modified NBA allowing testing of infused patients
  - Developed a means of confirming newly detected inhibitors using alternative testing methods
  - A proportion of low titer inhibitors are false positives.
FVIII Inhibitor Method Validation

Each modification to the Nijmegen-Bethesda assay was validated.

- Shipping on cold packs vs. frozen: n=50, r=0.998
- Use of commercial vs. locally prepared imidazole-buffered normal pool plasma: r=0.97
- Use of buffer instead of FVIII deficient plasma for dilution: n=71, r=0.99 (not adopted)
- Elimination of FVIII contamination:
  - Measurable FVIII in 126/228 (55%) of frozen specimens
  - Due to prophylaxis, episodic treatment, or ITI
  - If FVIII is not accounted for, residual activity will be 100% or higher leading to NBU of 0.
  - Instituted heating step, 56° for 30 minutes then centrifugation
  - Measured FVIII activity and FVIII antigen after heating, <1%

Change in Inhibitor Titer after Heating Plasma

Specimens with NBU <1.0

1/159 with negative history (0.6%) and 5/30 with positive history (16.7%) increased above cut-off, $P=0.0004$
Change in Inhibitor Titer after Heating Plasma

21 specimens with positive NBU

NBU (Heated specimens) vs. NBU (Unheated specimens) with a correlation coefficient of r=0.94.
# Modified Nijmegen-Bethesda Method

**Heat patient and control plasmas to 56°C and centrifuge.**

Dilute patient plasma in *FVIII-deficient plasma,* if an inhibitor is expected.

<table>
<thead>
<tr>
<th>Patient Mix</th>
<th>Control Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 part patient or dilution</td>
<td>1 part <em>FVIII-deficient plasma</em></td>
</tr>
<tr>
<td>plus 1 part imidazole-buffered normal pooled plasma (BNPP)*</td>
<td>plus 1 part imidazole-buffered normal pooled plasma (BNPP)*</td>
</tr>
</tbody>
</table>

Incubate for 2 hours at 37°C

Measure Factor VIII activity

Patient mix/control mix X 100 = % residual activity (RA)

Convert RA to NBU by formula

Adjust for dilution, if necessary

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*Nijmegen modifications

**CDC modification

 Controls:  
Negative control CV 9.8% (n=117)  
Positive control CV 10.3% (n=114)
Cutoff of $\geq 0.5$ for positive inhibitor misclassified fewer specimens than a cut-off of $\geq 0.6$. 

FVIII Inhibitor Cut-off

a. At enrollment ($n=644$)

b. All specimens ($n=1259$)
**FIX Inhibitor Assay**

- **Heating:**
  - 1 of 17 specimens changed with heating (0 to 0.6 NBU)

- **Establishment of inhibitor cut-off:**
  - All with negative history of inhibitor had NBU ≤0.2.

- **Controls:**
  - Negative control: CV = 6.8%
  - No positive control

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![Graph showing the distribution of NBU values for enrollment and all specimens.](image-url)
Inhibitor Method Comparison

- 3 methods used in HIRS:
  - Functional clot-based assay:
    - Modified Nijmegen-Bethesda assay (NBA)
  - Functional chromogenic assay:
    - Identical to NBA except FVIII method (CBA)
  - Antibody detection method:
    - Fluorescence immunoassay (FLI)
Inhibitor Method Comparison

<table>
<thead>
<tr>
<th>NBA Result (n)</th>
<th>CBA Negative n (%)</th>
<th>CBA Positive n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBA Negative (883)</td>
<td>880 (99.7)</td>
<td>3 (0.3)</td>
</tr>
<tr>
<td>NBA Positive (122)</td>
<td>37 (30.3)</td>
<td>85 (69.7)</td>
</tr>
<tr>
<td>≥ 2.0 NBU (42)</td>
<td>0</td>
<td>42 (100)</td>
</tr>
<tr>
<td>0.5-1.9 NBU (80)</td>
<td>37 (46.2)</td>
<td>43 (53.8)</td>
</tr>
</tbody>
</table>

- 1005 specimens compared in NBA and CBA
- 37 specimens (4%) were NBA positive and CBA negative, all 0.5-1.9 NBU.
  - 5 had positive DRVVT
  - 13 had non-time-dependent inhibition
Fluorescence Immunoassay (FLI) for Antibodies to Factor VIII

Performing on 272 specimens
Sensitivity: 0.03 NBU

FLI positive in:
- 98% of CBA positive specimens
- 82% of NBA positive specimens
Comparison of Chromogenic and FLI Results to Clotting NBU in Study Specimens

<table>
<thead>
<tr>
<th>Nijmegen-Bethesda Units</th>
<th>Chromogenic</th>
<th>FLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0-0.4</td>
<td>21%</td>
<td>100%</td>
</tr>
<tr>
<td>0.5-0.9</td>
<td>43%</td>
<td>100%</td>
</tr>
<tr>
<td>1.0-1.9</td>
<td>50%</td>
<td>72%</td>
</tr>
<tr>
<td>2.0-4.9</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>≥5.0</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
Comparison of Chromogenic and FLI Results to Very Low Titer Clotting NBU

![Bar chart comparing Chromogenic and FLI results to Nijmegen-Bethesda Units](chart.png)
Inhibitor Method Comparison

Conclusions:

- NBA, CBA, and FLI agree on specimens ≥2.0 NBU.
- FVIII specificity could not be demonstrated for 26% of inhibitors <2.0 NBU using 2 tests with different mechanisms.
- Low titer inhibitors detected in clot-based assays should be repeated by testing a new specimen and confirmed in tests more specific for FVIII.
- 21% of NBA-negative patients have anti-FVIII antibodies.

Immune Tolerance Induction (ITI) Therapy Monitoring

- 38 patients who had been on ITI in HIRS
- Positive FLI was present in:
  - 0 of 15 patients with successful ITI
  - 5 of 5 patients with failed ITI
  - 61% of 18 patients with ongoing ITI
- Differs from previous reports of positive antibodies after successful ITI
- Suggests that this might be a useful test for monitoring ITI
Low Titer Inhibitor Method

- NBA modified to increase sensitivity
  - Patient plasma concentrated
  - Ratio of plasma to normal pooled plasma 3:1 instead of 1:1
  - Uses chromogenic assay to measure Factor VIII
- Limit of detection 0.03 Bethesda units
- Identifies presence of inhibitors after successful ITI which reduce half-life and recovery (n=7)

U.S. Inhibitor Surveillance Testing

Modified Nijmegen-Bethesda Assay

- <0.5 NBU
  - Re-evaluate next year
- 0.5-1.9 NBU
  - Confirmatory Tests CBA, FLI, DRVVT
- ≥2.0 NBU
  - Test Repeat Specimen
    - <0.5 NBU
    - ≥0.5 NBU
      - Incident Case Data Collection
Inhibitor testing: 646 specimens

- Elevated inhibitor titer frequency
  - Hemophilia A: 42/506, 8%
  - Hemophilia B: 3/140, 2%
  - VWD Type 3: 1/8, 13%
Inhibitor “Outbreak” Investigation

- 4 new inhibitors in hospitalized previously treated patients over 14 months - ? product-related

- Investigation compared:
  - 2 time periods (no inhibitors previous 3 years)
  - Patients developing and not developing inhibitors while hospitalized

- Conclusions:
  - Incidence did increase
  - Not product-related
  - Inhibitor patients had more factor and more hospital days, increased odds of infection, continuous infusion, and product switch
  - A specific cause could not be determined.

Ghaji et al. Manuscript in preparation