Laboratory testing for the diagnosis and monitoring of therapy in hemophilia

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Disclosures for David Lillicrap

In compliance with COI policy, ISTH requires the following disclosures to the session audience:

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<th>Bayer, Bioverativ, CSL, Octapharma</th>
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<tr>
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Presentation includes discussion of the following off-label use of a drug or medical device: 

<N/A>
The Hemostatic Cascade
Intrinsic Tenase Complex

FIXa

FX

FXa

FVIIIa

Ca^{2+}

Ca^{2+}

Activated Phospholipid Surface Platelet Endothelium WBC
Intrinsic Tenase Complex in Hemophilia A

FIXa

FX

200,000-fold Reduced Catalytic efficiency

Ca^{2+}
Factor VIII Functional Assays

- One-stage assay
- Chromogenic assay
- Two-stage assay
Use of Factor VIII Assays

Potency labelling of FVIII products (Industry):

Accurate potency labelling of factor concentrates critical for product dosing

No labelling of protein mass – ie. specific activity

Kitchen et al. Haemophilia 2014; 20:36-42
Use of Factor VIII Assays

Potency labelling of FVIII products (Industry):

Potency and specific activity are critical attributes that define a particular product:

a) consistency of production

b) concentrate efficacy

Kitchen et al. Haemophilia 2014; 20:36-42
Use of Factor VIII Assays

Clinical measurement of FVIII:

Quantifying FVIII levels is important for the diagnosis of hemophilia A

Considerations –

a) Level of sensitivity

b) Assay discrepant hemophilia A

Kitchen et al. Haemophilia 2014; 20:36-42
Clinical Use of Factor VIII Assays

- At diagnosis
- At times of interventions - eg. surgeries
- Pharmacokinetic determinations - trough levels
- Apparent resistance to factor replacement
Measurement of FVIII for Clinical Management

• FVIII-specific assays
  
  • FVIII:C  a) one-stage  
  b) chromogenic

• FVIII:Ag

• Global hemostatic assays
  
  a) Thrombin generation  
  b) Thromboelastrography
Effect of anti-hemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a simple one-stage anti-hemophilic factor assay procedure.

Effect of anti-hemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a simple one-stage anti-hemophilic factor assay procedure.

Fig. 2. Graph illustrating method of plotting data for calculation of relative AHF activity the one-stage assay (Legend from original Fig. 2 on page 642 in the Journal of Laboratory and Clinical Medicine 41: 1953).
One-stage FVIII clotting assay - APTT

- FDA recommends the one-stage clotting assay to assign concentrate potency

- Simple and easy to perform, readily automated and more commonly used for monitoring

- Initiation of coagulation, FVIII activation, and subsequent FXa, thrombin and fibrin generation occurs in a “single reaction” thus termed “one-stage”

Barrowcliffe TW, Hubbard AR, Kitchen S. Haemophilia. 2012; 18. 61-65
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid

Recalcify
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid

Recalcify

Time to Clot
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid

- Chemically-depleted
- Immuno-depleted
- Naturally deficient
- +/-VWF
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid

- Micronized silica
- Ellagic acid
- Kaolin
- Cellite
One-stage FVIII:C Assay

Test PPP + FVIII deficient PPP + Activator + Phospholipid

- Total PL
- % PS
Survey of FVIII Assays Used

There is extreme diversity in the reagents and assay conditions used for the one-stage method:

In clinical laboratories in the UK the following are used

- 25 different APTT reagents
- 20 different FVIII deficient plasmas
- 13 different reference plasmas
- 11 different instruments

The chromogenic method has less variability with only 5 different kits from 4 manufacturers available

Barrowcliffe TW. Haemophilia 2006;12:23-29
Buffer for Dilution

- 4% HBS-BSA
- FVIII-deficient plasma
- HemosIL Factor Diluent
- HemosIL Factor Diluent + 1% Albumin
- Imidazole
- Michaelis buffer
- NaCl 0.9%
- Owrens-Koller
- Owrens-Koller Veronal
- Saline
- Stago Diluent
- Veronal
One-stage clotting assay - APTT

Timer starts with the addition of calcium to initiate the reaction and stops when the reaction reaches a preset increase in one of the following:

- Turbidity (optical measurement)
- Viscosity (mechanical measurement)

One-stage FVIII Assay limitations (1)

Considerable inter- and intra laboratory variation due to –

- Different instrument platforms
- Choice and handling of reference material
- Misleading when assaying for potency of rFVIII products

One-stage Factor VIII Assay Limitations (2)

Large variability due to:

- Sources of clotting activators, phospholipids and factor deficient plasma may differ, kaolin activators are too dense for optical analyzers
- Susceptible to interference from preactivation of FVIII generated during venipuncture
- Interference from anti-phospholipid antibodies
Chromogenic FVIII assay

• The European Pharmacopoeia recommends use of the chromogenic-based assay in order to assign concentrate potency

• Most clinicians in Europe and North America rely on the one-stage APTT-based assay in the management of their patients

Chromogenic FVIII assay

- Direct determination of FVIII cofactor activity:
  - FVIII mediates FXa generation

- More expensive, more precise and robust, but narrower dynamic range:
  - Standard range: 20% to 150% (Chromogenix kit)
  - Low range: 1% to 20% (Chromogenix kit)

Chromogenic FVIII:C Assay

Test PPP  +  FIXa  +  FX  +  IIa
Chromogenic FVIII:C Assay

Test PPP + FIXa + FX + IIa

↓

FXa
Chromogenic FVIII:C Assay

Test PPP  +  FIXa  +  FX  +  IIa

↓
↓
↓

FXa

↓

FXa Substrate  →  Color
Chromogenic FVIII Assay Limitations

• Expensive?

• Short reagent shelf life

• Limited number of manufacturers

A survey from a European external quality assessment program (ECAT) for laboratories in the field of hemostasis performed in 2013 found the following:

- 193/214 (90.2%) used the one-stage assay
- 13/214 (6.1%) used the chromogenic assay
- 8/214 (3.7%) used the two-stage clotting assay
Factor Assays and New Hemophilia Products

Bioengineered Recombinant Factor Concentrates

Single Chain FVIII
PEGylated FVIII and FIX - Significant one-stage assay variability
FVIII & FIX Fc Fusions

Potential Solutions

• Product specific one-stage assay standard

• One-stage assay result “conversion factor”

• Chromogenic factor assays
Factor Assays and New Hemophilia Products

Non-Factor Replacement Therapies

- FVIII mimetics (Emicizumab)
- Rebalancing hemostasis strategies (Fitusiran, Concizumab etc)

Potential Laboratory Tests for Monitoring

- Global hemostasis assays - Thrombin generation assays/Thromboelastography
- FVIII chromogenic assays (human reagents) for Emicizumab
Discrepancies of FVIII Assay Results in Non-Severe Hemophilia A

~30% of non-severe HA patients

One-Stage FVIII > Chromogenic - more frequent
(~15% of non-severe HA may be missed)

Chromogenic > One-Stage

Which assay best reflects the clinical bleeding phenotype - Unresolved
Discrepancies of FVIII Assay Results in Non-Severe Hemophilia A

1. One-Stage FVIII > Chromogenic
   Mutations at A domain interfaces – increased FVIIIa instability

2. Chromogenic > One-Stage
   Mutations at thrombin cleavage and FIXa binding sites
One-Stage Chromogenic

Chromogenic One-Stage

F8 Mutations Resulting in FVIII Assay Discrepancies
Laboratory Testing for Factor VIII Inhibitors

Anti-FVIII antibody generation (IgG₄/IgG₁)

Proceedings: A more uniform measurement of factor VIII inhibitors


The Bethesda Assay

114 Centers
Median Inhibitor: 5 BU
Range: 0-64 BU
CV 106%
(39% with 1 outlier removed)

FVIII Inhibitor Testing EQA

Jennings et al. Haemophilia 2009
Molecular Genetic Testing for Hemophilia
Isolation and characterization of a cDNA coding for human factor IX

(cDNA hybridization/DNA sequence analysis/blood coagulation)

KOTOKU KURACHI AND EARL W. DAVIE

Department of Biochemistry, University of Washington, Seattle, Washington 98195

Contributed by Earl W. Davie, July 29, 1982

and George Brownlee, Oxford University

Factor IX Gene Cloned in Nov 1982
Characterization of the human factor VIII gene


Departments of Molecular Biology and *Protein Biochemistry, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, USA

The complete 186,000 base-pair (bp) human factor VIII gene has been isolated and consists of 26 exons ranging in size from 69 to 3,106 bp and introns as large as 32.4 kilobases (kb). Nine kb of mRNA and protein-coding DNA has been sequenced and the mRNA termini have been mapped. The relationship between internal duplications in factor VIII and evolution of the gene is discussed.

and Genetics Institute

Factor VIII Gene Cloned in Nov 1984
Spectrum of Hemophilia Mutations

> 2,100 different $F8$ mutations
http://hadb.org.uk/WebPages/PublicFiles/MutationSummary.htm

> 1,100 different $F9$ mutations
http://www.factorix.org/
Types and Patterns of Hemophilia Mutations

- Severe hemophilia A - 50% due to 2 recurrent inversion mutations
- Hemophilia B - more CRM+ mutants
- Recurrent, founder mutations in non-severe hemophilia
National Hemophilia Genetic Testing Programs

1. Canada
   >4,000 diagnostic reports in past 17 years

2. USA
   My Life Our Future Project: >5,000 patients diagnosed
Factor VIII Intron 22 Inversion
45% of Severe Hemophilia A

Test for using either long-range PCR or inverse PCR strategies
Molecular Testing for Inherited Bleeding Disorders

1. Confirmation of uncertain phenotypic diagnosis
2. Molecular analysis as the preferred diagnostic test
3. Supplementary genotype-phenotype information
4. Differentiation of genocopies
Molecular Testing for Inherited Bleeding Disorders

Confirmation of the phenotypic diagnosis

Mild Quantitative Deficiencies

eg. mild FIX/FVIII deficiency
Molecular Testing for Inherited Bleeding Disorders

Molecular Analysis as the Preferred Diagnostic Test

a) Prenatal diagnosis

a) Carrier detection
Unusual Test Samples

Tsar Nicholas II, Tsarina Alexandra and Family
The Royal Hemophilia Mutation
Rogaev et al. Science October 2009

CTCAAAAG-ATC

F9 Intron 3 Splice Acceptor Mutation
Royal Family F9 Splicing Mutation
Molecular Testing for Inherited Bleeding Disorders

Differentiation of Genocopies

(identical phenotypes due to mutations in different genes)
Differentiation of Genocopies
(identical phenotypes due to mutations in different genes)

2. Type 2N VWD vs Mild/Moderate Hemophilia A

Genetic Locus: \( \text{VWF} \) vs \( \text{F8} \)

Therapy: VWF/FVIII concentrate vs FVIII concentrate
DDAVP vs DDAVP
Molecular Testing for Inherited Bleeding Disorders

Supplementary Genotype – Phenotype Information

- FVIII/FIX Inhibitor risk
- Hemophilia B Leiden
<table>
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<th>Genotype</th>
<th>Risk (%)</th>
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<tr>
<td>Multi-domain deletions</td>
<td>~60-80%</td>
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<tr>
<td>Light chain nonsense mutns</td>
<td>30-40%</td>
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<tr>
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<td>20-25%</td>
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<td>Single domain deletions</td>
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<td>Heavy chain nonsense mutns</td>
<td>10-20%</td>
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<td>FVIII missense mutns</td>
<td>&lt;10%</td>
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<tr>
<td>Small A run insertions/deltns</td>
<td>&lt;5%</td>
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<tr>
<td>Splicing mutns.</td>
<td>&lt;5%</td>
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Enhanced Inhibitor Risk F8 Mutations in Non-Severe Hemophilia A

Inhibitor Risk at 50 EDs

- Trp2229Cys - 41.7%
- Arg2159Cys - 39.4%
- Asp2074Gly - 21.2%
- Arg593Cys - 18.3%
Post-Pubertal Changes to F.IX in Normal Subjects and Hemophilia B Leiden Patients

Graph showing the increase in FIX with age for normal subjects and Hemophilia B Leiden FIX patients.
Hemophilia B Leiden Mutations

Gene

5'  -220  -190  -26  -21  -20  -19  -6  -5  +6  +8  +11  +13  3'

DBP  C/EBP

AR  HNF4  COUP-TF  ARP  C/EBP
Queen’s University in Kingston