Molecular Testing for \textit{RHD} on patients with serologic weak D phenotype

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BloodCenter of Wisconsin

Objectives

• Discuss the benefits of using a molecular approach in testing patients with a serologic weak D phenotype.
• List the methods used to perform \textit{RHD} genotyping.
• Describe an approach for phasing in \textit{RHD} genotyping for transfusion medicine practice.

\textbf{RH Genes – Rh Positive}

Chromosome 1

Locus 1 - presence of \textit{RHD} codes for the presence of D or no D.
Locus 2 - presence of \textit{RHCE} codes for Ce, CE, cE, ce.

\textbf{RhD Protein}

Vestibule

• Crosses RBC membrane 12 times
• No sugars attached

\textbf{Terminology Guidelines for RH Alleles}

• D positive - \textit{RHD} conventional sequence is assigned \textit{RHD*01}
• D negative phenotypes (\textit{RHD} null) designated with N and the background allele on which the null mutation has occurred
  • Common Rh negative - \textit{RHD*01N.01}
  • 37 bp insertion - \textit{RHD*01N.02}

http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/

http://www.jic.ac.uk/corporate/about/publications/advances/images_10/protein.jpg
Terminology Guidelines for RH Alleles

- Alleles encoding partial D are given numbers associated with the phenotype category or classification.
- Alleles encoding partial DVI are numbered as RHD*06.
- DVI types encoded by different alleles numbered sequentially as RHD*06.01, RHD*06.02.

http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/

Terminology Guidelines for RH Alleles

- Weak D alleles are designated by RHD*01W.01 (type 1) OR numbers begin with 100.
- Weak D phenotypes described with anti-D in numerous cases relative to their prevalence, the terminology "weak partial D" is used to reflect both weak D RBC typing and risk for anti-D production.

http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/

Serologic Weak D Phenotype Definition

- Anti-D reagent giving no or weak (≤2+) reactivity in initial testing, but agglutinating moderately or strongly with antihuman globulin.
- Identified by weak reactivity or by discordant typing results.


Weak Expression of D 2 Categories

- Not at risk of making anti-D
- At risk of making anti-D

Weak Expression of D Not at Risk of Making Anti-D

- Weak D “Types”: amino acid change(s)
  - Usually single change
  - Types 1, 2, 3 most common in Caucasians
Weak D Types
Fewer Copies of Rh Protein

- Antigens/RBC*
  - Type 1: 759
  - Type 2: 491
  - Type 3: 1948

Normal RhD antigen

Weak Expression of D
At Risk of Making Anti-D

- Partial Ds: hybrid RHD alleles
- Weak partial D
- DEL: detected by adsorption/elution
- D epitopes on RHCE gene

Partial D
Missing RhD Epitopes

Normal RhD antigen
Partial D
Altered RhD Epitopes

Normal RhD antigen

D Epitope on RHCE Gene - ceHAR

ceHAR results from one RHD exon inserted into the RHCE gene.

D Epitope on RHce Gene - ceCF

ceCF results from 3 nucleotide changes, 48G>C, 697C>G, 733C>G in RHce gene.

Gene Conversion

• Portions of RHCE into RHD

Gene Conversion

• Portions of RHD into RHCE

Missense, Nonsense, Frameshift & Splice Site Mutations

Missense – amino acid change
Nonsense or Frameshift – prevent expression
Splice site – no or reduced expression
**RH Gene Diversity**

- **RHD**: > 200 alleles causing weak expression of RhD antigen
- Weak D “Types” - >135 alterations
- Partial D - >100 alterations
- DEL - >40 alterations

Frequency of Serologic Weak D phenotypes – estimated 2.9% among mixed population in USA

**Commentary**

It’s time to phase-in RHD genotyping for patients with a serologic weak D phenotype


**Recommendation of the Work Group**

- “RHD genotyping is recommended whenever a weak D phenotype is detected by routine Rh blood typing of pregnant women and other females of childbearing potential.”
- Strong Recommendation: based on high-quality evidence from observational studies (1A)

**Unnecessary RhIG Injections in USA**

- 3,953,000 Live Births
- 3,812,000 Pregnancies
- 556,500 RHD-negative
- 16,700 Serologic Weak D
- 13,360 weak D types 1, 2, or 3

24,700 Unnecessary ante- & postpartum RhIG

**Reasons to Resolve Weak Expression Pregnancy**

- Avoid giving RhIG to women who do not need it (Rh status is confirmed for historical discrepancies)
- Resolve early in pregnancy to eliminate false-positive rosette tests
“Testing for “weak D”, formerly “Du” antigen is being recommended in a new approach due to new genotyping capabilities and information. They can be genotyped and if their genotype is type 1, 2 or 3, they may be managed as RhD-positive…”

Additional Notes

- CPT code 81403 for RHD genotyping (Tier 2 Molecular pathology procedure, Level 4)
- Reimbursement rates for the Tier 2 code established
- ACOG updating its Practice Bulletin!!

Unnecessary RhD-Negative Transfusions in USA


5,000,000 Individuals Transfused Annually

730,000 RhD-negative

21,900 Serologic Weak D

Could receive RhD-positive units (47,700)

RHD Genotype

Weak and partial D management in patients

Which techniques do you use for RHD genotyping?

- PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)
- PCR with (Allele or) Sequence Specific Primers (PCR-SSP)
- Bead Technology (RHD BeadChip)
- BioChip Technology (RHD BloodGen)
- Sanger Sequencing of genomic DNA and cDNA
- Exon Scanning Assays
PCR with Allele-Specific Primers

- Isolate DNA
- Allele-specific amplification by PCR
  - Target weak & partial D variants
- Amplified products visualized on gel electrophoresis

Bead Chip Arrays

- Probe
  - AGGCTGCA
  - CGACTGCT

DNA Sequencing

- Gold standard for mutation detection
- Determines precise order of nucleotides
- Any method or technology used to determine order of the four bases—adenine, guanine, cytosine, and thymine—in DNA strand
- Next Generation sequencers simplify sequencing of genomes (introns & exons)
- Analyze many genes at one time

Identifying When to Perform RHD Genotyping

- AABB/CAP Work Group Recommendations
  - Anti-D reagent giving no or weak (\(\leq 2+\)) reactivity in initial testing, but agglutinating moderately or strongly with antihuman globulin
  - Discordant result with history or between reagents

Identifying When to Perform RHD Genotyping

- Two Method Strategy
  - Gel Test
    - Monoclonal Blend Anti-D
  - Test Tube
    - Monoclonal/Polyclonal Blend Anti-D

RHD Reference Sequence


Monoclonal Reagent Types

- Blend of monoclonal & polyclonal antibodies
- Blend of two or more monoclonal antibodies, each secreted by a different cell line
- IgG or IgM, or combination of IgG + IgM

Why?
- D antigen has >30 different epitopes
- Variant D antigens

FDA Approved Reagent Anti-D - Tubes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immucor-4</td>
<td>MS201</td>
<td>MS26</td>
</tr>
<tr>
<td>Immucor-6</td>
<td>TH28</td>
<td>MS26</td>
</tr>
<tr>
<td>Ortho Bioclone Tube</td>
<td>M4D2</td>
<td>Human polyclonal</td>
</tr>
<tr>
<td>Bio-Rad Seraclone - Blend</td>
<td>BS221</td>
<td>BS221</td>
</tr>
<tr>
<td>Bio-Rad Seraclone - 226</td>
<td>BS226</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Alpha</td>
<td>LGM1</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Beta</td>
<td>LGM3</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Delta*</td>
<td>LGM1</td>
<td>EDS1-M</td>
</tr>
<tr>
<td>Quotient (Alba) – Blend</td>
<td>LGM3</td>
<td>EDS1</td>
</tr>
</tbody>
</table>

*Not for patient testing, detects DVI at IS

FDA Approved Reagent Anti-D - Other Methods

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<thead>
<tr>
<th>Anti-D</th>
<th>Method</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immucor – Series 4</td>
<td>Galileo Echo®/Neo®</td>
<td>MS201</td>
<td>MS26</td>
</tr>
<tr>
<td>Immucor – Series 5</td>
<td>Galileo Echo®/Neo®</td>
<td>TH28</td>
<td>MS26</td>
</tr>
<tr>
<td>Ortho</td>
<td>GetProvue®</td>
<td>MS201</td>
<td></td>
</tr>
<tr>
<td>PK2</td>
<td>PK200/PK300®</td>
<td>P3X81</td>
<td></td>
</tr>
<tr>
<td>Blend</td>
<td>PK200/PK300®</td>
<td>P3X81</td>
<td>P3X200</td>
</tr>
<tr>
<td>Bio-Rad 226</td>
<td>Tango®</td>
<td>BS226</td>
<td></td>
</tr>
<tr>
<td>Bio-Rad 232</td>
<td>Tango®</td>
<td>BS232</td>
<td></td>
</tr>
<tr>
<td>Solidscreen II Blend (weak D testing)</td>
<td>Tango®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ortho</td>
<td>DG Get/Erytre®</td>
<td>P3x81</td>
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Monoclonal IgM/IgG ANTI-D

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<tr>
<td>Blend</td>
<td>PK200/PK300®</td>
<td>P3X81</td>
<td>P3X200</td>
</tr>
<tr>
<td>Bio-Rad 226</td>
<td>Tango®</td>
<td>BS226</td>
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</tr>
<tr>
<td>Bio-Rad 232</td>
<td>Tango®</td>
<td>BS232</td>
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<td>Ortho</td>
<td>DG Get/Erytre®</td>
<td>P3x81</td>
<td></td>
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</table>

Monoclonal IgM/IgG ANTI-D #1

Direct Agglutination - IS

Two Method Strategy

Criteria for RHD Genotyping

1. Gel at least 2+ or greater than tube test result
2. <2+ with both methods or ≤2+ with gel only or ≤1+ with test tubes only
3. Anti-D detected

Luo et. al, Blood Transfus DOI 10.20450/2017.0274-16
Two Method Strategy

Results
Luo et. al, Blood Transfus DOI 10.20450/2017.0274-16

• 50 cases
  • 14 retrospective
  • 36 prospective
• 32 women & 18 men
• 27 African American, 16 Hispanic, 6 Caucasian, 1 unknown

Two Method Strategy

RHD Genotyping Results
Luo et. al, Blood Transfus DOI 10.20450/2017.0274-16

• D variants identified in 49 cases
  • 39 partial D (RHD*DAR, DAU4, DAU5)
  • 10 weak D
  • Identified more partial Ds
  • Predictive Value – 98%

What is this donor’s RhD type?

• 27 y/o Caucasian donor typed O Rh Negative on 1st donation

• On 2nd donation PK7300 ® results Rh type as NTD

• Sample is sent to IRL for discrepancy resolution

What is this donor’s RhD type?

IRL Results

<table>
<thead>
<tr>
<th>Anti-D</th>
<th>IgM</th>
<th>IgG</th>
<th>IS</th>
<th>IAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-Clone™ Gamma401</td>
<td>F8D8</td>
<td>0</td>
<td>W+</td>
<td></td>
</tr>
<tr>
<td>Immucor-5</td>
<td>TH28</td>
<td>MS26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ortho Biocline™ MAD2</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Biotest (Bio-Rad) Blend</td>
<td>BS232</td>
<td>BS221</td>
<td>H41 11B7</td>
<td>0</td>
</tr>
</tbody>
</table>

What is this donor’s RhD type?

IRL Results

Anti-D          | IgM | IgG | IS | IAT |
----------------|-----|-----|----|-----|
Gamma-Clone™    | F8D8| 0   | W+ |
Immucor-5       | TH28| MS26| 0  |
Ortho Bioclone™ | MAD2| Human| 0 |
Biotest (Bio-Rad) Blend | BS232 | BS221 | H41 11B7 | 0 | W+ |

What is this donor’s RhD type?

Weak D type 2 allele

Weak D type 2 allele

Weak D type 2 allele

Weak D type 2 allele
**What is this donor’s RhD type?**

<table>
<thead>
<tr>
<th>RBCs Tested</th>
<th>Anti-C</th>
<th>Anti-E</th>
<th>Anti-c</th>
<th>Anti-e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Pos Ctl.</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Neg Ctl.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Rh Phenotype – D+C+E+c+e+
Probable Rh Genotype – *DCE/DcE*

*Most Probable - DcE/dCe*

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**What is this donor’s RhD type?**

- Weak D Type 2 Allele & RHCE*Ce likely in trans
- Further decreasing RhD expression

Blood Donor – Rh Positive
Prenatal Patient – Rh Positive
Transfusion Recipient – Rh Negative

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**Objectives**

- Discuss the benefits of using a molecular approach in testing patients with a serologic weak D phenotype.
- List the methods used to perform RHD genotyping.
- Describe an approach for phasing in RHD genotyping for transfusion medicine practice.

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**Thank You!!**

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Celebrating 70 Years