Serologic Weak D Phenotype to RH D Genotyping: How will I know? Part 1
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Director, Clinical Education
BloodCenter of Wisconsin

Commentary
It’s time to phase-in RH D genotyping for patients with a serologic weak D phenotype
Transfusion 2015 Mar;55(3):680–9

How will I know?
Objectives
• Identify causes for variability of expression of RhD antigen
• Describe specificity, sensitivity & intended use of current commercial RhD typing reagents used in test tube & automated methods.
• List challenges of typing for RhD by serologic methods.
• Define management & transfusion options for patients with weak or variable RhD typing.
**Variables Impacting Rh Typing**

<table>
<thead>
<tr>
<th>Suppliers of Variability</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD Gene</td>
<td>Weak D</td>
</tr>
<tr>
<td></td>
<td>C in Trans to RHD</td>
</tr>
<tr>
<td></td>
<td>Partial D</td>
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<td></td>
<td>D&lt;sub&gt;det&lt;/sub&gt;</td>
</tr>
<tr>
<td>D epitopes on RHCE Protein</td>
<td>cecF</td>
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<tr>
<td></td>
<td>ceMnAR</td>
</tr>
<tr>
<td>Anti-D Reagents</td>
<td>Polyspecific</td>
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<tr>
<td></td>
<td>Slide and Modified Tube</td>
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<tr>
<td></td>
<td>Human IgG</td>
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<td></td>
<td>Monoclonal IgG</td>
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<td>Monoclonal IgM</td>
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<td></td>
<td>Monoclonal Human IgG</td>
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<td>Blends</td>
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<tr>
<td>Testing Platform</td>
<td>Test Tubes</td>
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<td></td>
<td>Column Agglutination</td>
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<td></td>
<td>Solid Phase</td>
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<td>Liquid</td>
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<td>Monotiter</td>
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<tr>
<td>Individual being Rh Typed</td>
<td>Transfusion Recipient</td>
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<td></td>
<td>Obstetrical Patient</td>
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<tr>
<td></td>
<td>Cord Blood</td>
</tr>
<tr>
<td></td>
<td>Donor Blood</td>
</tr>
</tbody>
</table>

Transfusion Technology Report Vol. #013 Immucor, Inc.
RhD Negative

- Deletion of RHD
- Inactivating mutations of RHD
  - RHD$\psi$ in African Americans
- Hybrid RHD-CE-D in African backgrounds

RH Genes in Rh Negative Caucasians

Chromosome 1

Locus 1 deletion of RHD therefore, no D antigen.
**RH Genes in Rh Negative - African Background**

Chromosome 1

- **Locus 1**
  - RHCE

- **Locus 2**
  - RH

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
</table>
| Exons | No D antigen | C/c and E/e antigens

- **Locus 1** – 37 bp insertion & several mutations in **RHD** results in no product

**RHD** Allele frequency of ~ .030 African Americans*

*Reid ME, et al Blood Cells, Molecules & Diseases 2013


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**Rh (D) Negative – African Background**

Chromosome 1

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>10</th>
</tr>
</thead>
</table>
| Exons | No D antigen | C/c and E/e antigens

- **Locus 1** – **RHCE** inserted in **RHD** results in no D antigen and weak C.

**Allele frequency of hybrid RHD-CE-D is ~ .029-.09**

*Reid ME, et al Blood Cells, Molecules & Diseases 2013


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**What About Weak Expression of D?**
Traditional Weak D Reactivity with Anti-D

- Agglutinated with some anti-D on direct agglutination (IS)
- Negative on direct agglutination (IS)
  - D antigen detected by IAT only

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

Serologic Weak D Phenotype
Definition

- Anti-D reagent agglutinates RBCs weakly (≤ 2+) or not at all by test tube method at IS, but agglutinating moderately or strongly with antihuman globulin

  Identified by weak reactivity or by discordant typing results
Frequency of Serologic Weak D

- RhD-negative individuals
- Estimated to be 2.9% among a mixed population

Weakened Expression of D 2 Categories

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

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

- C in \textit{trans} with \textit{RHD}
  (Ceppellini effect)
  - \(r'\) haplotype (\(R1r' - Dce/Ce\))

- Weak D “Types”: amino acid change(s), usually a single change
  - Types 1, 2, 3

Weak D Types
\textit{Most Not At Risk of Making Anti-D}

- Changes in regions of \textit{RHD} predicted to be in the \textbf{RBC membrane or inside RBC}
- Less Rh protein in RBC membrane
- Can type as Rh-positive or Rh-negative by direct agglutination with monoclonal (IgM) anti-D reagents

<table>
<thead>
<tr>
<th>Type</th>
<th>IS</th>
<th>Anti-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>(w^+ - 2^{+})</td>
<td>Anti-D</td>
<td>0</td>
</tr>
<tr>
<td>IS</td>
<td>D IAT</td>
<td>C IAT</td>
</tr>
<tr>
<td>Anti-D</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Account for 80-90\% of Weak D
Not at risk of making Anti-D

- Type 1 Val(270)Gly
- Type 2 Gly(385)Ala
- Type 3 Ser(3)Cys
Normal RhD Positive
Normal Rh Protein

~ Antigens/RBC*
DcE/ce 9900-14,600
DcE/ce 12,000-19,700
DcE/DcE 14,500-22,800

*Daniels G. Human Blood Groups

Weak D Type 1- 81
Fewer Copies of Rh Protein

~ Antigens/RBC*
Type 1 759
Type 2 491
Type 3 1948

*Blood 2000;95:2699-2708

WEAK EXPRESSION OF D
At Risk of Making Anti-D
Weak Expression of D At Risk of Making Anti-D

- Partial Ds: hybrid RHD alleles
  - DVI
  - Others…
- D<sub>el</sub>: detected by adsorption/elution
- D epitopes on RHCE gene

Partial D

- Lack exofacial epitopes or have altered exofacial epitopes
  - Hybrid proteins
  - Missense mutations affecting exofacial protein

Partial D Missing RhD Epitopes

Normal RhD antigen
Partial D
Altered RhD Epitopes

Partial D – European Ancestry
- DNB, DVI and DVII most common in European ancestry
- DVI
  - 2 reports of fatal hydrops fetalis
    - Transfusion. 1983 Mar-Apr;23(2):91-4
- Anti-D reagents designed to be neg at IS/pos at IAT

Partial D – African American
- DIIIa & DIVa most common
  - Type as RhD positive at IS

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
<th>D IAT</th>
<th>Cl. IAT</th>
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<tbody>
<tr>
<td>Anti-D</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>3+</td>
</tr>
</tbody>
</table>
Deletion of exon 9 in Asians occurs in 10-30%.

D<sub>el</sub>
- Type as D-negative (IS & IAT), only adsorb & elute anti-D
- Severely reduced protein

D Epitope on RHCE Genes
- ceHAR - formally known as R<sup>H</sup><sub>0</sub>Har or D<sup>H</sup>Har
- ceCF (ceRT, ceSL) - Crawford phenotype

D Epitope on RHCE Gene - ceHAR

ceHAR results from one RHD exon inserted into the RHCE gene.
ceHAR Phenotype: Reactivity with Reagent Anti-D

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Anti-D</th>
<th>RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Gamma-Clone</td>
<td>GAMA401</td>
<td>FBD8</td>
</tr>
<tr>
<td>Immucor-4</td>
<td>MS201</td>
<td>MS26</td>
</tr>
<tr>
<td>Immucor-5</td>
<td>TH28</td>
<td>MS26</td>
</tr>
<tr>
<td>Ortho Biclon</td>
<td>MAD2</td>
<td>Human</td>
</tr>
<tr>
<td>Ortho (ID-MTS)</td>
<td>MS201</td>
<td></td>
</tr>
<tr>
<td>Biotest (Bio-Rad)</td>
<td>BS232</td>
<td>BS221</td>
</tr>
<tr>
<td>Quotient - Alpha</td>
<td>LDM1</td>
<td></td>
</tr>
<tr>
<td>Quotient - Delta</td>
<td>LDM1</td>
<td>ESD1M</td>
</tr>
</tbody>
</table>

*Positive reactions often weaker at IAT

D Epitope on RHce Gene - ceCF

No D antigens

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

Anti-D Reagents: Reactions with Crawford Phenotype RBCs

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Anti-D</th>
<th>RBCs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IgM</td>
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</tr>
<tr>
<td>Quotient - Delta</td>
<td>LDM1</td>
<td>ESD1M</td>
</tr>
</tbody>
</table>
Anti-D Reagents & Methods

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>
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<tbody>
<tr>
<td>Gamma-Clone</td>
<td>GAMM401</td>
<td>FD8</td>
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<td>Immucor-4</td>
<td>MS201</td>
<td>MS26</td>
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<tr>
<td>Immucor-5</td>
<td>TH28</td>
<td>MS26</td>
</tr>
<tr>
<td>Ortho Biocline Tube</td>
<td>MAM2</td>
<td>Human polyclonal</td>
</tr>
<tr>
<td>Bio-Rad Seracrine - Blend</td>
<td>BS232</td>
<td>BS221</td>
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<tr>
<td>Bio-Rad Seracrine - 226</td>
<td>BS226</td>
<td></td>
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<tr>
<td>Quotient (Alba) – Alpha</td>
<td>LDM1</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Beta</td>
<td>LDM2</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Delta*</td>
<td>LDM1</td>
<td>ESS11</td>
</tr>
<tr>
<td>Quotient (Alba) – Blend</td>
<td>LDM3</td>
<td>ED51</td>
</tr>
</tbody>
</table>

*Not for patient testing, detects DVI at IS
FDA Approved Reagent Anti-D - Other Methods

<table>
<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>
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</tr>
<tr>
<td>PK1</td>
<td>PK7200®/PK7300®</td>
<td>P3X61</td>
<td></td>
</tr>
<tr>
<td>PK2</td>
<td>PK7200®/PK7300®</td>
<td>HM10</td>
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<td>Blend</td>
<td>PK7200®/PK7300®</td>
<td>P3X61</td>
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<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>
<td></td>
</tr>
<tr>
<td>Solidcreen II Blend (weak D testing)</td>
<td>Tango®</td>
<td>H411B7</td>
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<tr>
<td>Grifols</td>
<td>DG Gel/Erytra®</td>
<td>BS221</td>
<td></td>
</tr>
</tbody>
</table>

Specificity of Anti-D
CFR 660.26

- Panel of antigen positive RBCs must be tested and include at least 3 donors of each phenotype:
  - DCE/ce, DCE/ce
  - dCE/ce, dCE/ce
  - Group A, B and O dce/dce
  - Most include testing with DVI

Sensitivity of Anti-D

- Reagent Anti-D compared to Reference Blood Grouping Sera (FDA) for potency
- Monoclonal Anti-D WILL NOT detect all weak D and partial D
- Weak D Test (IAT/AHG) enhances reactivity with most examples of weak D and partial D
Monoclonal IgM/IgG ANTI-D #1
Weak D Test - IAT

Monoclonal IgM/IgG ANTI-D #2

Method Variability
• Test Tube Methods
  • Reagents used
  • Technologist-dependent
  • Weak D IAT/AHG or not…
• Other Methods
  • Liquid microtiter
  • Column Agglutination
Direct Agglutination in Test Tubes

Reading Direct Agglutination
ABO vs. RhD Reagents

<table>
<thead>
<tr>
<th></th>
<th>Anti-A / Anti-B</th>
<th>Anti-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Class</td>
<td>IgM</td>
<td>IgM or IgM &amp; IgG</td>
</tr>
<tr>
<td>Antigen Structure</td>
<td>Carbohydrate</td>
<td>Integral Membrane Protein</td>
</tr>
<tr>
<td>Antigen Sites/RBC</td>
<td>(~2 \times 10^5)</td>
<td>(~9,900 \sim 33,000)</td>
</tr>
</tbody>
</table>

Reading Direct Agglutination

Shake, Rock, Roll, Swirl, Tilt, or some combination...

Grade as soon as RBC button is resuspended
Microplate ABO/Rh Typing
Fluid Phase – Hemagglutination by Settling

PK7300

Settling Method

Neg  Pos

Microplate ABO/Rh Typing
Fluid Phase – Hemagglutination

Galileo Echo® & NEO®

Microtiter Plate ABO/Rh Typing
Hemagglutination Method

Monoclonal Control
Anti-A
Anti-B
Anti-D Series 4
Anti-D Series 5
A1 Cells
B Cells
Microplate ABO/Rh Typing
Hemagglutination by Agitation

Column Agglutination
Hemagglutination Methods

Questionable RhD Typing Results Using Automation

<table>
<thead>
<tr>
<th>Microplate</th>
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<tbody>
<tr>
<td>Anti-D</td>
<td>? Or NTD</td>
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</table>

<table>
<thead>
<tr>
<th>Gel</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>&lt;2+</td>
</tr>
</tbody>
</table>

or
Discordant with historical typing
RhD Typing Results
Test Tube Method

<table>
<thead>
<tr>
<th>Tube</th>
<th>IS</th>
<th>IAT</th>
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</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>0-2+</td>
<td>2-4+</td>
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</table>

or

<table>
<thead>
<tr>
<th>Tube</th>
<th>IS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>3-4+</td>
</tr>
</tbody>
</table>

Consider RHD genotyping

Reasons to Resolve Weak Expression in 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

Rosette Test Results

- Negative Control
- Positive Control
- Weak D+ Mom

Courtesy of MR Combs
Reasons to Resolve Weak Expression Transfusion

- Conserve Rh-negative blood for D-negative recipients (high risk of making anti-D)

Objectives

- Identify causes for variability of expression of RhD antigen
- Describe specificity, sensitivity & intended use of current commercial RhD typing reagents used in test tube & automated methods.
- List challenges of typing for RhD by serologic methods.
- Define management & transfusion options for patients with weak or variable RhD typing.
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- Flegel WA. Molecular genetics and clinical applications for RH. Transfusion and Apheresis Science 2011;44:81-91.
- Garratty G, Glynn SA, McEntire for the Retrovirology Epidemiology Donor Study. ABO and Rh(D) phenotype frequencies of different racial/ethnic groups in the United States. Transfusion 2004;44:703-6.
Thank You
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