Serologic Weak D Phenotype to RHD Genotyping: How will I know?

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BloodCenter of Wisconsin

How will I know?
Objectives
• Identify at least 3 different causes for variability of expression of the RhD antigen
• List the challenges of typing for RhD by serologic methods
• Define the management of pregnant women and transfusion options for patients who present with weak or variable RhD typing

How will I know?
Objectives
• Discuss the recent recommendations of the Inter-organizational Work Group on RHD genotyping for managing pregnant women and transfusion recipients who have a serologic weak D phenotype.
### Variables Impacting Rh Typing

<table>
<thead>
<tr>
<th>Contributors of Variability</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD Gene</td>
<td>Weak D, C in Trans to RHD, Partial D, DEL</td>
</tr>
<tr>
<td>D epitopes on RhCE Protein</td>
<td>cecF, cehAR</td>
</tr>
<tr>
<td>Anti-D Reagents</td>
<td>Polyspecific Slide and Modified Tube Human IgG, Monoclonal IgG, Monoclonal IgM, Monoclonal IgM, Monoclonal Blends</td>
</tr>
<tr>
<td>Testing Platform</td>
<td>Test Tubes S &amp; IAT, Column Agglutination, Solid Phase, Liquid Monolayer</td>
</tr>
<tr>
<td>Individual being Rh Typed</td>
<td>Transfusion Recipient, Donor Blood, Donor Blood, Cord Blood</td>
</tr>
</tbody>
</table>

Transfusion Technology Report Vol. #013 Immucor, Inc.

### RH Genes – Rh Positive

**Chromosome 1**

- **Locus 1**
  - RHD: RHCE
  - Locus 1: Presence of RHD codes for the presence of D or no D.

- **Locus 2**
  - RHCE: RHCE
  - Locus 2: Presence of RHCE codes for Ce, CE, ce, cE.

### RhD Protein

- Vestibule
- Crosses RBC membrane 12 times
- No sugars attached

[Image of protein structure with annotations]
RhD differs from RhCE by 34 to 37 amino acids.

RhD Negative
- Deletion of *RHD*
- Inactivating mutations of *RHD* in African Americans
- Hybrid *RHD*-CE-*D* in African backgrounds

Gene Conversion
- Portions of *RHCE* into *RHD*
Gene Conversion

• Portions of RHD into RHCE

Missense, Nonsense, Frameshift & Splice Site Mutations

RHD

5' G>C 3'

RHCE

3' C>G 5'

Missense – amino acid change
Nonsense or Frameshift – prevent expression
Splice site – no or reduced expression

RH Gene Diversity

• RHD: > 200 alleles
  • Weak D “Types”
  • Partial D
  • DEL
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


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 *trans* with *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**

*Most Not At Risk of Making Anti-D*

- Changes in regions of *RHD* predicted to be in the **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>IS</th>
<th>D IAT</th>
<th>Cl IAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>w+</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

---

Account for 80-90% of Weak D

*Not at risk of making Anti-D*

Adapted from Flegel, Curr Opin in Hemat 2006, 13:476–483
### Normal RhD Positive

Normal Rh Protein

<table>
<thead>
<tr>
<th>Antigens/RBC*</th>
<th>DcE/ce</th>
<th>9,600–14,600</th>
</tr>
</thead>
<tbody>
<tr>
<td>DcE/ce</td>
<td>12,000–19,700</td>
<td></td>
</tr>
<tr>
<td>DcE/DcE</td>
<td>14,500–22,800</td>
<td></td>
</tr>
</tbody>
</table>

*Daniels G. Human Blood Groups*

### Weak D Type 1- 81

Fewer Copies of Rh Protein

<table>
<thead>
<tr>
<th>Antigens/RBC*</th>
<th>Type 1</th>
<th>759</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 2</td>
<td>491</td>
</tr>
<tr>
<td></td>
<td>Type 3</td>
<td>1948</td>
</tr>
</tbody>
</table>


### WEAK EXPRESSION OF D

At Risk of Making Anti-D
Weak Expression of D
At Risk of Making Anti-D

- Partial Ds: hybrid \textit{RHD} alleles
  - DVI
  - DIIIa
  - Others
- DEL: detected by adsorption/elution
- D epitopes on \textit{RHCE} gene

Partial D

- Lack exofacial epitopes or have altered exofacial epitopes
  - Hybrid proteins
  - Missense mutations affecting \textbf{exofacial protein}

Partial D
Missing RhD Epitopes

Normal RhD antigen
Partial D
Altered RhD Epitopes

Normal RhD antigen

D Epitope on RHCE Genes

- ceHAR - formally known as $R_{0}^{Har}$ or $D^{HAR}$
- ceCF ($ceRT$, $ceSL$) - Crawford phenotype

D Epitope on RHCE Gene - ceHAR

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

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons</td>
<td></td>
</tr>
<tr>
<td>1 2 3 4 5 6 7 8 9 10</td>
<td>Exons</td>
</tr>
<tr>
<td>No D antigens</td>
<td>ce antigens</td>
</tr>
</tbody>
</table>

Anti-D | 3+ | IS
ceHAR Phenotype: Reactivity with Reagent Anti-D

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Anti-D</th>
<th>IgM</th>
<th>IgG</th>
<th>ceHAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-Clone</td>
<td></td>
<td>GAMA401</td>
<td>F8D8</td>
<td>Pos*</td>
</tr>
<tr>
<td>Immucor-4</td>
<td></td>
<td>MS201</td>
<td>MS26</td>
<td>Pos*</td>
</tr>
<tr>
<td>Immucor-5</td>
<td></td>
<td>TH28</td>
<td>MS26</td>
<td>Pos*</td>
</tr>
<tr>
<td>Ortho Biodone</td>
<td></td>
<td>MAD2</td>
<td>Human</td>
<td>Neg</td>
</tr>
<tr>
<td>Ortho (ID-MTS)</td>
<td></td>
<td>MS201</td>
<td></td>
<td>Pos</td>
</tr>
<tr>
<td>Biotest (Bio-Rad)</td>
<td></td>
<td>BS232</td>
<td>BS221</td>
<td>Pos</td>
</tr>
<tr>
<td>Quotient - Alpha</td>
<td></td>
<td>LDM1</td>
<td></td>
<td>Pos</td>
</tr>
<tr>
<td>Quotient - Delta</td>
<td></td>
<td>LDM1</td>
<td>ESD1M</td>
<td>Pos</td>
</tr>
</tbody>
</table>

*Positive reactions often weaker at IAT

D Epitope on RHce Gene - ceCF

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Anti-D</th>
<th>IgM</th>
<th>IgG</th>
<th>Crawford</th>
</tr>
</thead>
<tbody>
<tr>
<td>GammaClone</td>
<td></td>
<td>GAMA401</td>
<td>F8D8</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td>Immucor-4</td>
<td></td>
<td>MS201</td>
<td>MS26</td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Immucor-5</td>
<td></td>
<td>TH28</td>
<td>MS26</td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Ortho Biodone</td>
<td></td>
<td>MAD2</td>
<td>Human</td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Ortho (ID-MTS)</td>
<td></td>
<td>MS201</td>
<td></td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Biotest (Bio-Rad)</td>
<td></td>
<td>BS232</td>
<td>BS221</td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Quotient - Alpha</td>
<td></td>
<td>LDM1</td>
<td></td>
<td>Neg/Neg</td>
</tr>
<tr>
<td>Quotient - Delta</td>
<td></td>
<td>LDM1</td>
<td>ESD1M</td>
<td>Neg/Neg</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>Anti-D</th>
<th>IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-Clone</td>
<td>GAM401</td>
<td>F8D8</td>
<td></td>
</tr>
<tr>
<td>Immucor-4</td>
<td>MS201</td>
<td>MS26</td>
<td></td>
</tr>
<tr>
<td>Immucor-5</td>
<td>TH28</td>
<td>MS26</td>
<td></td>
</tr>
<tr>
<td>Ortho Biocline Tube</td>
<td>MA02</td>
<td>Human polyclonal</td>
<td></td>
</tr>
<tr>
<td>Bio-Rad Seraclove - Blend</td>
<td>BS232</td>
<td>BS221</td>
<td>H4111B7</td>
</tr>
<tr>
<td>Bio-Rad Seraclove - 226</td>
<td>BS226</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Alpha</td>
<td>LDM1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Beta</td>
<td>LDM3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Delta*</td>
<td>LDM1</td>
<td>ESD1-M</td>
<td></td>
</tr>
<tr>
<td>Quotient (Alba) – Blend</td>
<td>LDM3</td>
<td>ED51</td>
<td></td>
</tr>
</tbody>
</table>

*Not for patient testing, detects DVI at IS
**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

Monoclonal IgM/IgG ANTI-D #1
Direct Agglutination - IS

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 – 10 x 10^5</td>
<td>~ 9,900 – 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>
<th>Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D</td>
<td>Anti-D</td>
</tr>
<tr>
<td>? Or NTD</td>
<td>? or ≤2+</td>
</tr>
</tbody>
</table>

or

Discordant with historical typing

http://www.grifols.com
www.ortho-wire.com/en/blood-group-serology/learning/learninglibrary/AgAb/page5.cfm
RhD Typing Results
Test Tube Method

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

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


http://www.aabb.org/advocacy/statements/Pages/statement150722.aspx
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)

Additional Notes

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

Protocols for High-Risk Pregnancies

p. 360

“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…”

**Result of RhD Typing by Manual Tube or Automated Methods in Initial Testing**

- **Negative**
  - Discrepant/inconclusive or strength of reaction weaker than expected (serologic weak D phenotype)
  - Candidate for RbG RhD-negative for transfusion

- **Positive (and consonant with patient history, if available)**
  - Not a candidate for RhG RhD-positive for transfusion

- **Weak D type 1, 2 or 3 Not detected**
  - May be at risk for forming anti-D
  - Candidate for RhG RhD-negative for transfusion

- **Weak D type 1, 2 or 3 Detected**
  - Not at risk for forming anti-D
  - Not a candidate for RhG RhD-positive for transfusion

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**Serologic Weak D Phenotype Detected**

- **Low Resolution**
  - Molecular testing appropriate for phenotype

- **High Resolution**
  - Molecular testing includes weak D type and partial D assays

- **Yes**
  - Molecular testing explains phenotype

- **No**
  - Sequencing

**Result Reported**

---

**RHD BeadChip**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Amino Acid Change</th>
<th>Nucleotide Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Adapted from Wagner, F. Perspectives in Transfusion Medicine, Issue 5, Grifols, 2015.
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
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

Unnecessary RhIG Injections in USA


- 3,953,000 Live Births
- 3,812,000 Pregnancies
- 556,500 RhD-negative
- 16,700 Serologic Weak D

24,700 Unnecessary ante- & postpartum RhIG

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)
Case 1

- 57 y/o female
- Diagnosis – dehydration, hypotension, anemia
- Patient is RhD positive with anti-D

Referred for Partial D Analysis

Case 1 - IRL Results
Test Tube Method

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
<th>IAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D – S-clone</td>
<td>0</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-D – Series 5</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>Anti-D – Gamma-Clone</td>
<td>0</td>
<td>4+</td>
</tr>
<tr>
<td>Anti-D – B-clone</td>
<td>0</td>
<td>4+</td>
</tr>
<tr>
<td>Rh Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*RHD Genotype - Weak D Type 4.2 (DAR)*

Case 1 Summary

- Weak D Type 4.2 (DAR)
- Partial D
  - At risk of making Anti-D (did!)
  - A candidate for RhIg (not anymore)
  - Treat as Rh negative for transfusion
Case 2

- 25 y/o female
- Prenatal Visit
- RhD Discrepancy observed

Referred for RhD Discrepancy Analysis

Case 2 - IRL Results
Test Tube Method

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
<th>IAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D – S-clone</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>Anti-D – Series 5</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>Anti-D – Gamma-Clone</td>
<td>0</td>
<td>2+</td>
</tr>
<tr>
<td>Anti-D – B-clone</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>Rh Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**RHD Genotype - Weak D Type 1**

Case 2 Summary

- Weak D Type 1
- RhD Positive
  - Not at risk of forming Anti-D
  - Not a candidate for RhIg
  - Treat as Rh positive for transfusion
Impossible to Know with Serology!

<table>
<thead>
<tr>
<th>All negative at IS</th>
<th>Case 1 Weak D Type 4.2</th>
<th>Case 2 Weak D Type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IAT</td>
<td>IAT</td>
</tr>
<tr>
<td>Anti-D – S-clone</td>
<td>4+</td>
<td>3+</td>
</tr>
<tr>
<td>Anti-D – Series 5</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>Anti-D – Gamma-Clone</td>
<td>4+</td>
<td>2+</td>
</tr>
<tr>
<td>Anti-D – B-clone</td>
<td>4+</td>
<td>3+</td>
</tr>
<tr>
<td>Rh Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

How will I know?
Objectives

• Identify at least 3 different causes for variability of expression of the RhD antigen
• List the challenges of typing for RhD by serologic methods
• Define the management of pregnant women and transfusion options for patients who present with weak or variable RhD typing

How will I know?
Objectives

• Discuss the recent recommendations of the Inter-organizational Work Group on *RHD* genotyping for managing pregnant women and transfusion recipients who have a serologic weak D phenotype.
References


References

• Flegel WA. Molecular genetics and clinical applications for RH. Transfusion and Apheresis Science 2011;44:81-91.

References

• Garlandt 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.
• Lacey PA, Caskey CR, Werner DJ, Moulds JJ. Fatal hemolytic disease of a newborn due to anti-D in an Rh-positive Du variant mother. Transfusion. 1983 Mar-Apr;23(2):91-4.
Thank You

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