Conflicts of Interest

The presenters have no conflicts to declare.

Resolving Typing Discrepancies Using All the Tools in the Toolbox

Effective use of test methods
Experience of the technologist and supervisor
Critical evaluation of the case history
Critical evaluation of the serologic and molecular test results

Cases will include discrepancies between historic and current typings, serologic typings with different reagents or methods, and discrepancies between genotype-predicted phenotype and serologic phenotype.

It's all about D

“Normal” D antigen – gene and protein normal
“Abnormal” D antigen
- Weak D: used to be defined as D+ by IAT only
  - **New Weak D definition in use now
- Partial D: differences in gene resulting in alterations in antigen expression
  - One very weak partial D:
    - DEL: D antigen detected only by adsorption and elution or molecular methods
- Increased D: D —
- RHD is an extremely polymorphic gene

Dr. Landsteiner Featured on the 1997 Austrian 1000 Schilling

Anti-D Reagent Information

With current monoclonal reagents:
- Most D+ RBCs react at Immediate Spin
- Many partial D RBCs react at Immediate Spin
  - Partial D status not known until anti-D detected
  - To detect partial D in D+, need molecular testing on D+ patients in the prenatal period or pretransfusion

With current monoclonal reagents:
- IgG component in tube test needed to detect D antigens reactive by AHG
- No single monoclonal anti-D reagent detects all D+ antigens
- Polyclonal anti-D tested by AHG phase detects most partial and weak D except Del
Serologic Testing

IRL Technician Carla Hands performs antigen testing.

Automated Typing Instruments for Patients

- ECHO
- IH-1000
- Erytra
- NEO
- Ortho Vision

Beckman PK7300 for Donor Testing

Weak D – Serologic Characterization

- Reported to be low D antigen copy number weakly reactive or requiring weak D test (indirect antiglobulin test previously called the D^- Test)
- Reactivity is varied
  - According to sample (#D sites per RBC)
  - According to method (sensitivity of test)
  - Most are detected by blood bank automation which detects ≥1+ weak D test (AHG phase in tube)
- <1% Caucasian population

Partial D - Serologic Characterization

- D antigen lacks defined epitope(s)
- Serologic Identification - historic
  - Monoclonal anti-D patterns identify the subtypes
  - Most partial D RBCs react with reagent anti-D at immediate spin phase
  - Most partial D RBCs detected by automated typing instruments
- Many partial D types at risk for alloimmunization to D

Anti-D Reagents

- Polyclonal (high protein)
- Monoclonal (low protein)
  - IgM monoclonal
  - Monoclonal blends
    - IgM monoclonal / IgM monoclonal
    - IgM monoclonal / IgG monoclonal
  - Monoclonal/Polyclonal blend
    - IgM monoclonal / IgG polyclonal
**D Testing – AABB BBTS Standards**

- AABB Std 5.8.2 - **Donors:** If the initial test with anti-D is negative, the blood shall be tested using a method designed to detect weak D
- AABB Std 5.14.2 - **Recipients:**
  - Rh type shall be determined with anti-D reagent
  - The test for weak D is unnecessary when testing the patient
- AABB Std 5.30.2.(3) - **Newborns of D− moms (RhiG candidates):** ...weak D testing is required when the test for D is negative

**D Testing Requirements AABB**

AABB Std 5.8.2 - **Donors:**
If the initial test with anti-D is negative, the blood shall be tested using a method designed to detect weak D

Blood Bank Automation approved by FDA for donor testing
detects donors with weak D test >1+ (by parallel AHG tube test)

- ∴ many weak D resulted as D+ in donors currently
- ∴ most partial D resulted as D+ in donors currently
- To detect all partial D, molecular testing all D+ and D− donors needed

**D Testing Requirements AABB**

AABB Std 5.14.2 - **Recipients:**
Rh type shall be determined with anti-D reagent. The test for weak D is unnecessary when testing the patient

- **Test method selected by facilities may yield final D interpretation differences in patients**
  - Some facilities type all patients by automation (detects most weak and partial D RBCs)
  - Some use tube testing with weak D (DAT phase) for all patients (detects more weak and partial D RBCs)
  - Some do not do weak D testing, only immediate spin as required

**D Interpreting Different if Donor or Patient**

- Autologous Donor presents for donation
  - Typed in donor center by Beckman PK7300 as D+
  - Patient Pre-surgical type and cross
  - Typed by Transfusion Service on Galileo as D+
- OR
  - Typed by BB in Tube Test as D negative, weak D test not done (not required) – interpretation D neg

- Autologous unit labeled D+, patient is resulted in BB computer as D-
  - Easily resolved with weak D test

**Implications for Weak D Patients**

- Selection of anti-D reagent/testing method important (some inserts say <2+ result is D negative)
- Weak D testing not required for transfusion recipients and prenatal patients, but **if not done**:
  - Weak D patients are resulted as D negative and receive D negative blood they may not require
  - Prenatal patients may be determined to be RhiG candidates and they may not require it

**Implications for Weak D Patients – cont.**

Weak D testing not required for transfusion recipients and prenatal patients, but **if done**:

- Weak D positives are resulted as either:
  - D+ and receive D+ blood OR
  - Weak D and receive D− blood or D+
  - (facility dependent)
- Prenatal patients are resulted as D+ and are not RhiG candidates (facility dependent)
- Accepted by ACOG (American College of Obstetricians and Gynecologists)
Implications for D negative Patients

- Selection of anti-D reagent/testing method important for donors and babies (automation vs. tube test)
- Detection of Weak D < 1+ by automation not required for donors ∴ some weakly reactive D antigens are not detected
- Weak D babies of D negative mothers may not be detected by automation, potential for D alloimmunization of the mother
  - Weak D detection required for babies of D negative mothers
  - Both expected to be very rare events, but will happen

Rh Discrepancies Caused by Variable Reactivity of Partial and Weak D types with Different Serologic Techniques*

- Monoclonal anti-D reagents did not distinguish between partial and weak D Types 1 and 2
- Weak D Types 1 and 2 do not show consistent reactivity with FDA-approved tube test reagents and technology
- Molecular tests that distinguish common partial and Weak D types provide the solution to resolving D antigen discrepancies
- To limit anti-D alloimmunization, it is recommended that samples yielding an immediate-spin tube test of 1+ agglutination or not more 2+ by gel technology be considered D− for transfusion and Rh immune globulin prophylaxis

Challenges in D Testing Interpretation

- Differences among FDA licensed reagents/methods may cause discrepancies between records:
  - Historical vs. current
  - Donor vs. recipient
  - Between facilities

Mitigation: Educate staff on reasons for discrepancies and when to investigate with molecular testing

Common Partial D – DVI

- Most common form of partial D in Caucasians, reactivity is like weak D:
  - Not detected at immediate spin
  - Usually detected at antiglobulin phase
  - Generally resulted as D neg as patients and D+ as donors
  - But one reagent - Alba Bioscience’s anti-D reacts at IS
    - ∴ ∴ ∴ ∴ ideal for testing donors but not for patients

Ant-D produced by DVI has resulted in fatal HDFN
- ∴ ∴ ∴ ∴ potential for clinical significance in pregnancy
- Treat as D neg for transfusion and RhIG prophylaxis
- Lack outcome data on RhIG effectiveness in partial D patients with D+ fetus/newborn

Reagent Reactivity with DVI RBCs

<table>
<thead>
<tr>
<th>DVI RBCs</th>
<th>Immediate Spin</th>
<th>Antiglobulin Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ortho GEL</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Ortho BioClone</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Gamma-clone blend</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Immucor 214</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Immucor Series 4</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Immucor Series 5</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Seraclone IgM</td>
<td>O</td>
<td>NA</td>
</tr>
<tr>
<td>Seraclone IgG antibody</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>Erytype 3 IgM</td>
<td>O</td>
<td>NA</td>
</tr>
<tr>
<td>Solidscreen II IgG</td>
<td>NT</td>
<td>NA</td>
</tr>
<tr>
<td>ALBAclone α &amp; β IgM</td>
<td>O</td>
<td>NT</td>
</tr>
<tr>
<td>ALBAclone B (IgM)</td>
<td>O</td>
<td>+</td>
</tr>
<tr>
<td>ALBAclone Δ IgM</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

Adapted from Regina Leger

FDA Requirements for DVI

- FDA requirement that monoclonal anti-D not detect partial DVI on immediate spin
- Goal to ensure that females of childbearing years who are of partial DVI type will be typed as D negative
- Donors with partial DVI type will type positive with weak D test
- It is the reason samples from pregnant women do not require weak D testing


Uncommon Partial D: DEL

- Del RBCs
  - Negative in routine IS and IAT tube tests
  - Negative in automated tests
  - Very low levels of D antigen detected by adsorption/elution or molecular methods only
  - Most frequently found in D– East Asians (~30%), rare in Europeans (different mutations)
  - Del donors type D-negative and when transfused to D– recipients, rare case reports of anti-D formed

Anti-D Crossreactivity

- RBCs with epitopes of D expressed on RHCE protein react strongly at immediate spin with some anti-D, these samples do not have D protein:
  - DHAR (formerly called RoHAR, better expressed as ceHAR)
    - German ethnicity
  - Crawford
    - African American ethnicity

Anti-D Crossreactivity - ceHAR

- Hybrid RHCE-D-CE gene, no RHD gene
- Rare, < 0.01%
- Routine high protein anti-D reagents usually do not detect
- ceHAR RBCs react strongly with some IgM anti-D
- ceHAR individuals can make anti-D
- Blood recipients with hybrid ceHAR gene should be given D negative RBCs
- Perhaps a reason to have different anti-D for patients and donors

Anti-D Crossreactivity – Crawford–Rh43

- Low incidence antigen – African American ethnicity (~0.1%)
- Rare allele RHCE*ceCF, no RhD protein
- Only a few reported anti-D are reactive:
  - anti-D clone GAMA401
  - Anti-D ALBAclone (may be reactive)
- Pregnant patients and transfusion recipients should be interpreted as D negative and receive RhIG and D negative blood

Anti-D Crossreactivity - ceHAR

<table>
<thead>
<tr>
<th>Immediate Spin</th>
<th>Gamma-clone</th>
<th>Immucor Series 4</th>
<th>Immucor Series 5</th>
<th>Ortho BionClone</th>
<th>Ortho Gel (ID-MTS)</th>
<th>Seraclone IgM</th>
<th>Seraclone blend</th>
<th>ALBAclone alpha &amp; beta IgM</th>
<th>ALBAclone blend</th>
<th>ALBAclone delta IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Adapted from Regina Leger

Common Partial D Reactivity and Anti-D Crossreactivity – Summary

<table>
<thead>
<tr>
<th>D type</th>
<th>As a Donor</th>
<th>As a Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVI</td>
<td>D+</td>
<td>D-</td>
</tr>
<tr>
<td>ceHAR, Crawford</td>
<td>D- *</td>
<td>D-*</td>
</tr>
<tr>
<td>Del</td>
<td>D+**</td>
<td>D-</td>
</tr>
</tbody>
</table>

*Difficult to manage electronically when anti-D typing sera is positive, but RBCs are not really D+
**Ideally, likely does not happen now
**Vox Sang International Forum**

**Genotyping for red blood cell polymorphisms**

Do you apply genotyping for RBC polymorphisms for the following:

- *RHD* genotype of the fetus to avoid unnecessary administration of anti-D immunoglobulin G (IgG) to D-negative pregnant women during pregnancy
  - If so: Do you use fetal DNA from the maternal plasma?
- Typing for the *RHD* genotype of the fetus only in cases in which the fetus is at risk for hemolytic disease

---

**Summary:**

- The fetal *RHD* genotype is determined in all participating countries if the fetus is at risk of anti-D hemolytic disease
- In the USA and Canada, DNA from amniotic fluid (amniocytes) is still used predominantly
- In the European countries and Brazil, cell-free fetal DNA isolated from maternal plasma is used for genotyping as well as amniotic fluid
- No countries routinely determine the *RHD* genotype of the fetus to prevent unnecessary prenatal administration of anti-D immunoglobulin

---

**Algorithm to Select Samples for Genotyping**

Current method in facility:

- Two typing for first time patients are performed with two methods
  - Gel and Tube (monoclonal/polyclonal blend at IS)
  - or Gel D

---

**Algorithm to Select Samples for Genotyping**

Three criteria used to determine if the sample should be sent for genotyping:

- Discrepancy between 2 methods and Gel reaction strength at least 2+ stronger than tube
- Serologic weak reaction strength <2+ regardless of testing method if both tube test and Gel performed
- Or <2+ if only Gel test performed
- Or <1+ if only tube test performed
- Presence of anti-D in D positive patient with no history of RhIg in last 3 months

---

**Algorithm to Select Samples for Genotyping**

A total of 50 patients ranging from newborn to 93 years in age were identified to be genotyped

- Genomic testing confirmed D variants in 49/50 cases
- Positive predictive value (PPV) of 98%
  - 1/50 (2%) was D+ with anti-D with 1 conventional *RHD* allele
  - auto-adsorption not performed
- Identified more partial D+ cases than other approaches
  - Identified 39/50 (78%) partial D+ cases

---

**Summary of Challenges of Serologic Typing**

- Serologic methods vary
  - Tube
  - Gel
  - Solid phase
- Serologic reagents vary or are unavailable
  - Monoclonal
  - Polyclonal
  - Blends
  - Patient source
- Simple (e.g. *Fy*<sup>a</sup>/Fy<sup>b</sup>) vs complex (e.g. RhD) epitopes
- Antigen variants can be missed (e.g. U variants)
- Expression level can hamper detection (e.g. weak D)
- Cross-reactivity (e.g. celHAR, ceCF)
### Molecular Testing Methods Differ in their Resolution

- **Low Resolution**
  - Gel-based methods
  - SSP-PCR for known SNPs
  - PCR-RFLP for known SNPs
  - High melt resolution analysis

- **Medium Resolution**
  - Arrays
  - Immucor BeadChip and Grifols IDCore
  - Taqman
  - MALDI-TOF

- **High Resolution**
  - DNA sequence analysis
  - Exon scanning
  - cDNA analysis
  - NextGen Sequencing

### RhD Variants

- **Weak Ds**
  - Alter quantity of protein, but not the surface D-epitope
  - Individuals do not usually make anti-D

- **Del**
  - Very weak expression of D, type D-
  - Can stimulate anti-D in D- patients

- **Partial Ds**
  - Have altered epitopes
  - Can type strongly D+
  - Can type weakly +
  - Often not recognized until they make anti-D

  **But it’s not always that clear cut!**

### Case 1: Background

- 24 year old Caucasian pregnant female
- Types D negative on ECHO with Immucor Series 4 and 5
- Types D w+ at immediate spin, 2+ at antihuman globulin
- Ordered RHD genotyping to resolve the discrepancy and determine candidacy for RhIg

### Case 1: RHD Genotyping

**Allele 1**

- RHD*01W.01 (weak D type)

  **Predicted Phenotype:** Weak D+

**Allele 2**

- RHD*01N.01

  **Predicted Phenotype:** Does not express RhD

Based on the RHD alleles identified, the patient is not at risk for production of allo-anti-D.

Rh Immunoglobulin not indicated for this patient.
Case 2: Background

- 40 year old Hispanic pregnant female
- Typed Group O D negative at outside lab
- Listed as O Positive in hospital records
- Current sample types
  - D negative on ECHO with Immucor Series 4 and 5 anti-D
  - D negative at immediate spin, 2+ at AHG
- Ordered RHD genotyping

Case 2: RHD Genotyping

<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*DAR</td>
<td>RHD*Dill-(CE4-7)-D</td>
</tr>
</tbody>
</table>

Expresses partial RhD

Does not express RhD

Predicted Phenotype: Partial D+

The patient is at risk for production of allo-anti-D

Characterization of the RHCE genes may be warranted

Case 2: RH Genotyping

<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*DIIa-CE(4-7)-D</td>
<td>RHD*DII-(CE4-7)-D</td>
</tr>
</tbody>
</table>

Expresses partial RhD

Does not express RhD

Predicted Phenotype: Partial D+, Altered C+, E- c+ e+ V+ VS+ hr-

The patient may be at risk for production of allo-anti-D and is at risk of allo anti-C, - c, - e, - hr

Case 3: Background

- 28 year old Caucasian female
- Pregnant
- Types D+ at immediate spin (w+ to 3+) with 3 of 4 sources of anti-D
- Typed D- at IS and at AHG with the fourth anti-D
- Requested RHD genotyping

Case 3: RH Genotyping

<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*weak partial RHD type 4.0</td>
<td>RHCE*ceHAR</td>
</tr>
</tbody>
</table>

Predicted Phenotype: D-, C-, E- c+ e+ Dhar+ Fppt+

The patient is not predicted to express D. They carry the RHCE*ceHAR allele

Individuals with a RHCE*ceHAR allele express D epitope(s) that type strongly D+ with some monoclonal anti-D reagents

Such individuals should be considered D negative

Anti-D Cross Reactivity – RHCE*ceHAR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Reactivity at Immediate Spin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-clone</td>
<td>+</td>
</tr>
<tr>
<td>Immucor Series 4</td>
<td>+</td>
</tr>
<tr>
<td>Immucor Series 5</td>
<td>+</td>
</tr>
<tr>
<td>Ortho BioClone</td>
<td>-D</td>
</tr>
<tr>
<td>Ortho gel (ID-MTS)</td>
<td>+</td>
</tr>
<tr>
<td>Seracclone IgM</td>
<td>+</td>
</tr>
<tr>
<td>Seracclone blend</td>
<td>+</td>
</tr>
<tr>
<td>ALBAclone alpha &amp; beta IgM</td>
<td>+</td>
</tr>
<tr>
<td>ALBAclone blend</td>
<td>+</td>
</tr>
<tr>
<td>ALBAclone delta IgM</td>
<td>+</td>
</tr>
</tbody>
</table>
Case 4: Background

- 25 year old Hispanic female, pregnant
- D typing discrepancy
  - Patient typed repeatedly D negative with negative Ab screen
  - Post-partum, both patient and infant type D negative at IS and positive (3+) at AHG
- Requested RHD variant workup

Case 4: Routine (targeted) testing

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nucleotide (Amino Acid)</th>
<th>Nucleotide(s) Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHD*01N.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RHD BEADCHIP™ Array includes 35 markers. Only nucleotides that differ from the consensus sequence are listed.

Probable RHD Genotype: RHD*01 / RHD*01N.01
Predicted RHD phenotype: D+

But this is not consistent with the reported serology!
Higher resolution testing is warranted.

Case 4: Revised Interpretation

<table>
<thead>
<tr>
<th>Allele 1 (Arg10Trp)</th>
<th>RHD*01W.61</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele 2</td>
<td>RHD*01N.01</td>
</tr>
</tbody>
</table>

Predicted Phenotype: weak D+

The patient was found to carry a variant not interrogated by the targeted RHD genotyping performed previously.

Due to the lack of information about the risk of alloimmunization in patients carrying this variant, it is recommended that this patient be treated as D negative for purposes of transfusion and a candidate for Rh Immunoglobulin.

Case 5: Background

- 19 year old female blood donor
- Jk(a+) typing discrepancy

- Donor typed Jk(a-) by serology
- Red cell unit distributed as Jk(a-)
- Hospital reported unit is XM incompatible
- Requested JK variant workup
Case 5: Targeted Genotyping

- Genomic DNA extracted from pilot tube
- DNA tested using PreciseType HEA Molecular BeadChip
  — Predicted donor to type Jk(a+b+)

<table>
<thead>
<tr>
<th>Kidd</th>
<th>Jka</th>
<th>Jkb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

But Remember Resolution?

- Low Resolution
  - Gel-based methods
  - SSP-PCR for known SNPs
  - PCR-RFLP for known SNPs

- Medium Resolution
  - Arrays such as BeadChip™

- High Resolution
  - DNA sequence analysis
  - Exon scanning
  - cDNA analysis
  - NextGen Sequencing

Case 5: High Resolution Genotyping

- Exon Scanning of coding exons

<table>
<thead>
<tr>
<th>Gene Region Method</th>
<th>Analytic Result</th>
<th>Variants detected</th>
<th>Predicted Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jk  gDNA seq* Exon 4</td>
<td>130G/A</td>
<td></td>
<td>E44K</td>
</tr>
<tr>
<td>Exon 5</td>
<td>None</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Exon 6</td>
<td>None</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Exon 7</td>
<td>588A&gt;G</td>
<td>silent</td>
<td></td>
</tr>
<tr>
<td>Exon 8</td>
<td>None</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Exon 9</td>
<td>838G/A</td>
<td>D280N</td>
<td></td>
</tr>
<tr>
<td>Exon 10</td>
<td>None</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Exon 11</td>
<td>None</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Case 5: High Resolution Genotyping

<table>
<thead>
<tr>
<th>Gene or Region Method</th>
<th>Analytic Result</th>
<th>Variants detected</th>
<th>Predicted Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jk (SLC14A1) c.838G/A (p.280Asp/Asn)</td>
<td>c.130G/A (p.44 Glu/Lys)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c.588A&gt;G</td>
<td>silent</td>
<td></td>
</tr>
</tbody>
</table>

Case 5: Assigning Alleles and Predicting Phenotypes

- JK*01W.01
  - c.838G (p.280Asp)
  - c.130G (p.44 Glu/Lys)
  - Synonymous SNP

- JK*02
  - c.588A>G
  - c.130G (p.44 Glu/Lys)
  - c.838G (p.280Asp)

Predicted Phenotype: Jk(a+ b+)

Genotyping identified a JK variant associated with weak antigen expression and typing discrepancies. The donor record should reflect the predicted phenotype.

Summary

- RhD variants are categorized as weak, partial and D del and in many cases serology cannot resolve the type and determine alloimmunization risk
- RHD genotyping can be used to resolve serologic weak D types not at risk for alloimmunization
- RHD genotyping can identify patients with partial D phenotype at risk to form allo-anti-D
- RBC genotyping can resolve typing discrepancies, and may require multiple molecular methods, with varying levels of resolution
Thank you for your attention!

Questions?

Sandra.Nance@redcross.org
Margaret.Keller@redcross.org