Molecular Testing in a Combined Transfusion & Donor Service

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Objectives:

- Review basic molecular blood typing technology and the rationale for its use
- Explain how molecular testing may benefit both transfusion services and blood donor centers
- Clarify recent publications on RHD molecular testing and the implications for transfusion medicine
Background: Adverse Reactions to Transfusion

- Hemolytic Transfusion Reactions (HTR)
  - Transfusion-Related Death
    - 3% ABO-related
    - 13% non-ABO-related

![Figure 1: Transfusion-Related Fatalities by Complication, FY2009 through FY2013](chart)

*Fatalities Reported to FDA Following Blood Collection and Transfusion. Annual Summary for Fiscal Year 2013.*
We routinely match RBCs for ABO and Rh with the intended patient. However, this means that minor RBC antigens are often incompatible, which can put the patient at risk for alloimmunization.

Some clinically significant alloantibodies (Jk\text{a}) will become senescent and less detectable with time, and can cause hemolytic events following even crossmatch compatible transfusions.

Alloimmunization rates are highly variable depending on the patient population (range 1% to about 60% [3]). Overall, the risk of delayed hemolytic transfusion reaction is estimated to be 1 in 2000 patients transfused, and the risk of a delayed serologic transfusion reaction 1 in 2500 patients transfused [5], indicating that alloimmunization remains a fairly common occurrence.
Background: Some patients at higher risk for alloimmunization

- Multiply transfused
- Autoimmune Hemolytic Anemia
- Multiparous females
- Transplant patients
Disadvantages

- Typing sera not available for all RBC antigens
- Result interpretation can be subjective
- Patients with +DAT: no direct agglutinating sera available
- Antibody source variation: poly vs monoclonal, human vs. other may affect performance
- Transfused patients: Problematic!
- Advanced serological techniques not always available
Background: Molecular Typing

- Antigens determined by multiple alleles defined by DNA sequence variations
- Allows prediction of the antigen phenotype
- PreciseType™ HEA first FDA Approved kit for RBC Molecular Typing
<table>
<thead>
<tr>
<th>Blood Group</th>
<th>RBC Antigens*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh</td>
<td>C (RH2), c (RH4), E (RH3), e (RH5), V (RH10), VS (RH 20)</td>
</tr>
<tr>
<td>Kell</td>
<td>K (Kel 1), k (KEL 2), Kpa (KEL3), Kpb (KEL 4), Jsa (KEL 6), Jsb (KEL 7)</td>
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<tr>
<td>Duffy</td>
<td>Fya (FY1), Fyb (FY2) GATA (FY-2), Fyx (FY2W)</td>
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<tr>
<td>Kidd</td>
<td>Jka (JK1), Jkb (JK2)</td>
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<tr>
<td>MNS</td>
<td>M (MNS1), N (MNS2), S (NS3), s (MNS4), Uvar (MNS-3,5W), Uneg (MNS-3,-4,-5)</td>
</tr>
<tr>
<td>Lutheran</td>
<td>Lua (LU1), Lub (LU2)</td>
</tr>
<tr>
<td>Dombrock</td>
<td>Doa (DO1), Dob (DO2), Hy (DO4), Joa (DO5)</td>
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<tr>
<td>Landsteiner-Wiener</td>
<td>LWa (LW5), LWb (LW7)</td>
</tr>
<tr>
<td>Diego</td>
<td>Dia (DI1), Dib (DI2)</td>
</tr>
<tr>
<td>Colton</td>
<td>Coa (CO1), Cob (CO2)</td>
</tr>
<tr>
<td>Scianna</td>
<td>Sc1 (SC1), Sc2 (SC2)</td>
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Basic Molecular Biology Review

- DNA contains four **nucleotides** that are linked together (base pair) to form the double helix structure.
- The nitrogenous bases are Adenine, Guanine, Cytosine, and Thymine. RNA contains Uracil.
- Using DNA as a template, complementary single stranded **mRNA** is synthesized via **transcription**.
- There are long stretches of DNA that contain both non-coding sequences (**introns**) and coding sequences (**exons**). mRNA is processed in the nucleus to remove the non-coding areas. Then the mature mRNA is transported to cytoplasmic ribosomes for protein synthesis.
Proteins are translated from mRNA by adding amino acid groups in a specific order determined by the codon sequence.

Twenty amino acids are specified by 64 codons (sets of 3 nucleotides).

Each codon is matched with a specific anticodon on a smaller RNA form, the transfer RNA (tRNA).

This is the central dogma of molecular biology. Genes are composed of DNA, which is Transcribed into RNA and Translated into Protein.
DNA sequence variations occur naturally in the population.

Many occur as only a single base difference (Single Nucleotide Polymorphism, or SNP).

There are approximately 10 million SNPs in the human genome. Some code for specific blood group antigens.

Types of DNA sequence variations:

- **Point mutations** substitute one nucleotide for another in the DNA.
- **Silent sequence variation.** More than one codon (a functional part of the three-letter genetic code) codes for the same amino acid. Has no effect on the resultant protein.
- **Insertions** add one or more extra nucleotides into the DNA sequence.
- **Deletions** remove one or more nucleotides from the DNA sequence.
- **Frameshift mutation** causes a shift in the reading frame (insertion or deletion) and may lead to an altered protein.
Unique on PreciseType HEA:

1. **Promoter silencing mutation for Fy\(^b\) (67T>C in \(FY\)),** giving a Duffy-null phenotype (also known as GATA mutation). These patients will safely tolerate Fy\(^b\) positive blood.

2. **Silencing mutations for S–s– phenotype, predicting Uvar or Uneg** antigen status (Intron 5 G>T and 230 C>T in \(GYPB\))


4. **RhC** based on three polymorphisms and the presence/absence of a 109bp insert in the **RHCE** gene, with indication of possible altered C antigen encoded by the (C)ce\(^s\) haplotype

5. **265C>T in \(FY\) gene, predicting Fy\(^x\)**, with varying degrees of weakened Fyb antigen, which may not always react with serologic reagents

6. **Hemoglobin S** marker (HgbS 173 A>T)
The PreciseType HEA System:

1. Synthetic beads are stained with unique colors.
   The beads are approximately 3 microns in diameter.
   Each unique color will correspond to an allele within the assay.

2. BioArray uses DNA oligonucleotides (probes) complementary to antigen specific alleles for multiple blood group systems.

3. The probes evenly cover the entire surface of a colored bead specific for that probe.
   Each bead has over 1 million copies of the probe.

4. BioArray Solutions
PreciseType Assay

- Multiplex PCR
  - DNA Amplification
  - Clean-up
    - Generate single stranded DNA (ssDNA)
  - Incubation on BeadChip
    - Amplicons bind to complementary DNA probe sequences on corresponding beads
• If the sequence of the amplified DNA perfectly matches to the 3’ end base, the probe is elongated, incorporating the fluorescent tag.

• If the amplified DNA does not perfectly match the probe, the DNA probe is not elongated, so the fluorescent tag is not incorporated.

• The Array Imaging System (AIS400) acquires an image of the chip with fluorescent (positive) and non-fluorescent (negative) beads.

• The assay image is converted to intensity data for analysis.
30K+ donors collected/year

Serve multiple hospitals in Indiana and surrounding states

- Pathology Staff
- Blood Supplier
- Clinical Lab, including Blood Bank testing
PreciseType Usage

- **Donors**
  - Group O, A and B donors
  - Donated >1X (encourage repeat donation)
  - Likely rare (African American, Amish)

- **Patients**
  - As needed

**Data Entry**

- Manual data entry into LIS (Millenium)
- Search ability with historic serologic and genotyping data
Encourage more interaction between recruiting staff and local groups that historically donate blood infrequently.
Who Benefits?

- The serologically complex patient
  - Warm autoantibodies and/or +DAT
  - High–titer low avidity antibodies, nonspecific antibodies
  - Multiple antibodies
  - Antibodies to high–frequency antigens
  - Patients with or with suspected antibodies for which no typing sera is available
Who Benefits?

- Chronically transfused patients
  - Antigen–matched RBC’s
  - Antigen–typed blood inventory
A Typical Case: Meet Bessie

• Bessie is a 75 year old patient in a smaller client hospital (<75 beds)

• Bessie’s transfusion and pregnancy history are not provided.

• Bessie is anemic (HGB 6.9 g/dL), and her hospital blood bank staff detected antibody(s) they could not identify, 2+ positive in both screen cells. 2 PRBC units are requested.

• We receive the sample, confirm that all screening cells in a standard panel are 3+ positive, as is the autocontrol. In our files, Bessie has a history of anti–E and uncomplicated transfusion of E–negative RBCs during orthopedic surgery in 2009.

• With both autoantibody and alloantibodie(s) in play, Bessie is a serologically complex patient. BioArray phenotype is initiated on the patient sample following confirmation of MD order.

• A Panel using PEG shows no added information, but a panel with no enhancement begins to show a pattern, suggesting both anti–E and possibly anti–c.

• At this point Bessie’s BioArray antigen profile is available, and this is forwarded to our reference laboratory along with available serologic results, history and sample.

• The reference laboratory confirms anti–E and anti–c are both present, and all other clinically significant alloantibodies are excluded using PEG autoabsorbed plasma (a technique not available in our laboratory). They recommend transfusing units negative for E, c, K, S and Jk^b based on the BioArray extended phenotype.
• So, here is where the real advantage comes in:

– Instead of pulling and screening dozens of units looking for the ‘right’ antigen combination, TMF techs go to the computer and search our phenotyped inventory for matching units.

– Selected units are then serologically confirmed. This saves substantial technical effort and reagents.

– Bessie’s phenotyped blood units are on their way much faster.

• This case also demonstrates another use of the BioArray genotyped inventory in our system: Identification of units when multiple antibodies are identified. Many times our genotyped inventory contains the units we need. This spares the expense of reference lab send-out.
A Second Case with an Interesting Observation

- 82 year old female, no transfusion history, 5 children

- Sent for antibody identification with request for 2 units PRBC

- Panagglutinating antibody(s) on panel: **Autocontrol 3+; DAT 3+**

- In saline with no enhancement: no reactivity

- BioArray initiated. Serologic RBC antigen typing also started to expedite processing

- **Patient RBC typing by Serology:**
  - D+
  - C+
  - c+
  - E+
  - e+
  - Kell +

- **Patient BioArray:**
  - D+
  - C–
  - c+
  - E+
  - e+
  - Kell–

RBCs coated with immunoglobulin will sometimes interfere with serologic tests.
Results from TMF Experience:

1. In our patient population, having this technology available has been very helpful in solving complex serologic cases. The availability of a genotyped inventory has decreased the time needed to identify antigen-matched blood and reduced send-out requests for antigen matched units.

2. Despite introduction of RBC genotyping test for clinical patients, it is not often requested by clinicians in our community practice medicine population. Currently we do not have standard “reflex orders” where blood bank staff can initiate molecular tests that are accepted by all hospital clients.

3. When genotyping would be helpful for complex compatibility problems, or the patient is chronically transfused and genotyping has not been ordered, TMF Blood Center Medical Director will consult with the treating physician to encourage test utilization where appropriate.

****An ongoing education issue with physician and hospital clients****
Blood Group Antigen Genetics

- As we have seen, many minor blood group antigens are encoded by single nucleotide changes, and are quite amenable to routine RBC genotyping.
- A and B antigens are serologically pretty uncomplicated, but genetically complex. More than 100 different alleles have been identified for the glycosyltransferases responsible for the four ABO types.

What about Rh? While serologic testing for the common Rh antigens D, C/c and E/e is fairly straightforward in most populations, antigen expression is more complicated in some ethnic groups. There are more than 200 \textit{RHD} alleles for weak D or partial D, and more than 100 \textit{RHCE} alleles for altered, hybrid proteins.
BioArray™ RHCE and RHD BeadChip™

RHCE and RHD Variant Typing by DNA Analysis

**RHCE Assay Variant Coverage**

- Crawford; VS; V; (C)ceCF; (C)ceS-1, 2, 3; 16C; ce; Ce; cE; CE; ce variant-1, 2; ceAR; ceAR CF; ceBl; ceEK; ceFV; CeMA; ceMO; ceRA; ceRT; ceS (340)-1, 1.1, 2, 2.1; ceS (697) (ceCF)-1, 1.1, 2, 2.1; ceS (748)-1, 1.1, 2, 2.1; ceS-1, 1.1, 2, 2.1; ceSL; ceTl type 2; ceTl-1, 2; CeVA; CeVG; CW-1, 2; CX-1, 2; DHAR-1, 2; E type I, III (EFM), IV; EKH; rN

**RHD Assay Variant Coverage**

- **Weak D type:** 1, 1.1, 2, 3, 4.0, 4.1, 4.2/DAR, 4.3, 5, 11, 14, 15, 17, 25, 29, 34, 40, 47, 51
- **D negative:** RHD; W16X; D-CE(3-7)-D; D-CE(4-7)-D; (C)dce; D-CE(3-9)-D; CE(1-3)-D(4-10); rG; RHD(Y269X)
- **Dq:** 1227 G>A; IVS3+1G>A; M295I
- **Partial D:** DAR/weak D 4.2; DIIIa (DIII type 5); DIII type 4.6; DIIIc; DIVa; DIVa -2; DIV type 3, 4, 5; DIVb; DV type 1, 2, 3 (DBS-0), 4, 5 (DHK), 6, 7, 8, 9; DVI type 1, 2, 3, 4; DNB; DHMi; DUC-2; DAU 1, 2, 3, 4, 5; DBT 1, 2; DCS 1, 2; DOL; DOL-3; DFR; DFR -2; DTO; DBS-0, 1, 2
• **RHD** and **RHCE** Genes are 97% identical and each have 10 exons that encode proteins which differ by 32-35 amino acids. RHD and RHCE proteins are 416 amino acids.

• **RHCE** encodes C/c and E/e antigens on a single protein. C/c differ by 4 amino acids, but only one is extracellular. E/e differ by one amino acid.

**Chromosome 1p34-1p36**
15% Hybrid RHD-CE-D with weak, altered C and e, VS expression AND no D (ce*)

Insertion of Premature Stop codon  66% South African,  
24% D negative African American (RHDψ pseudogene)
Gene Conversion Between RHD and RHCE gives Hybrid Genes of Partial D

Highly Homologous *Rhesus Boxes* (9kbps)

*SMP1 (small membrane protein)* seven exon gene
Weak D History

Described by Stratton (1946)

Defined as RBCs giving no or weak (≤2+) reactivity initially, but agglutinating moderately to strongly with antihuman globulin.

Formerly called ‘Du antigen’; renamed Weak D in 1992

Samples detected as “weak D” depend on the typing sera and method used. Different sera can lead to Rh typing discrepancies
Caused by DNA sequence variations that encode transmembrane or intracellular parts of the D protein that reduce surface D antigen expression.

Inability to sensitize and make alloanti-D is not (never was) included in the definition of weak D types.

Most Weak D patients do NOT make anti-D, but there are exceptions: Weak D types 4.2, DAR, 11,15, 21, and 57.

Weak D types 1 – 3 have not been reported to make alloanti-D.

- In persons with European ancestry, est. 0.2–1% are weak D phenotypes, and 90% are WEAK D ARE TYPES 1–3.
Partial and Category D

- Arise from hybrid *RHD/RHCE* genes and missense mutations to regions of *RHD* encoding parts of D external to RBC membrane

- React weakly with some monoclonal anti-D reagents because of an altered or missing epitope

- May make anti-D to ‘missing’ epitope of D
  - If transfused – should receive Rh-negative RBCs
  - If pregnant – are candidates for RhIG
Blood Group Variation: Common RhD Variants

- **Weak D**: Quantitative
  - Decreased number RhD surface
  - 76+ classified (point) mutations

- **Partial D**: Qualitative
  - Altered RhD epitopes
  - 70+ classified mutations, many hybrid alleles
Current rationale for D genotyping

- Weak and Partial D types cannot all be resolved by serology

- Rh negative blood is a limited resource (~15% population). Conserve Rh-negative blood for D-negative recipients with higher risk for making alloanti-D

- Avoid the administration of RhIG to women who do not need it
AABB and CAP convened a Work Group and charged it with developing recommendations to clarify the clinical issues related to RhD typing in persons with serologic weak D phenotype.

Recommended *RHD* genotyping whenever a serologic weak D phenotype and/or a discordant Rh D type is detected in patients, including pregnant women, newborns, and potential transfusion recipients.

Weak D types 1, 2, 3 can be managed as Rh Positive for the purposes of RhIG prophylaxis and selection of units for transfusion.

For Weak D other than 1, 2 or 3, treat as Rh negative: conventional RhIG prophylaxis, RhD negative for transfusion.

Work Group did not further address management of Partial D patients (but likely more information will follow....)
Weak D genotyping recommendations

- **Negative**
  - Candidate for RhIG
  - RhD-negative for transfusion

- **Discrepant/inconclusive or strength of reaction weaker than expected (serologic weak D phenotype)**
  - Send for RHD genotyping for weak D types

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

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

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

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Fig. 3. Algorithm for resolving serologic weak D phenotype test results by RHD genotyping to determine candidacy for RhIG and RhD type for transfusions.
Impact on RhIg resources

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- and postpartum RhIG injections

RHD Genotyping

Impact on Rh neg blood supply

5,000,000 Individuals Transfused Annually in US

730,000 RhD-negative

21,900 Serological Weak D

17,520 weak D types 1, 2, or 3

Could receive RhD-positive units (47,700 units)

RHD Genotyping

Serologic and genotyping data are complementary technologists...One does not replace the other

Genotyping is limited and some genetic variants may not be detected by the test.

An undetected mutation coding for silent or variant antigen expression may preclude clear distinction of alloantibody vs. autoantibody

Discrepancies between serologic and molecular RBC typing should be investigated and may require DNA sequencing at a reference laboratory.
Questions?
References