Molecular Testing in the Blood Bank

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Outline

• Current blood bank testing
  • Serology
  • Hematocrit
  • Hematocrit
• Molecular testing
  • What is this and how does it work?
  • Clinical uses of molecular testing
    • Prenatal testing
      • DNA testing
      • RHD zygosity
      • RHD-weak-D and Partial-D (Rh immune globulin treatment)
    • Providing an antigen-matched RBC units
      • Blood cell donation
      • Blood bank testing
      • Patient genotyping
      • Hematocrit
      • Hematocrit
• Current blood bank testing
  • For over 100 years, blood bank immunohematologic testing has relied on red cell agglutination and the formation of aggregates.
  • Antibodies, either IgG or IgM, bind to their corresponding red blood cell antigens and, either directly or indirectly, cause agglutination of those red blood cells.

Current Blood Bank Testing

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Current Blood Bank Testing

• Originally, this testing was done on tiles or in test tubes:

  ![Original Blood Bank Testing](image)

• Initially, antibody was isolated from donors who had high levels of specific antibodies, e.g., anti-D, anti-E, anti-Kell, or anti-c.

• But, varying strength, lack of stability, and limited availability ultimately led to the development and use of monoclonal antibodies—a process that is unlikely to continue due to the expense of developing new cell lines particularly for rare antisera.

Current Blood Bank Testing

• With the ongoing need to improve sensitivity and specificity—and the need to enhance turnaround times, larger blood banks and transfusion services began to use newer techniques to detect red cell agglutination:

  **Gel Columns:** Solid Phase Adherence Assays:

Current Blood Bank Testing

• In spite of increased sensitivity and some increase in objectivity with gel-testing and solid-phase techniques, these are still serologic techniques that rely on red cell agglutination—or at least the adherence of red cells to specific antibodies.

• As such they continue to share the same weaknesses:

  • Lack of specific antisera (even in the era of monoclonal antibodies the cost of developing new cell lines may limit the availability of additional new antisera)
  • False-positive results with some antisera (notably anti-M and anti-N)
  • Inability to discriminate between certain variant blood group antigens (weak-D vs. partial-D, E-antigen variants)
  • Inability to type RBC antigens in patients with a positive DAT, or patients with autoantibodies (without time-consuming absorption/elution techniques)
  • Typing patients who have been recently transfused and carry “2 RBC populations” (circulating donor and patient RBCs)
Conceptual Summary: Serologic Testing to Molecular Testing

DNA → Transcription → RNA → Translation → Protein → Post-Translation → Antigen

Serologic Testing

Molecular Testing

What makes this possible is the following:

- There are over 270 serologically determined blood group antigens, but the majority of genes have been identified, sequenced, and genetic polymorphisms assigned to blood group antigen specificities have been identified.
- The most common genetic variation responsible for differences in blood group antigens are coding region single nucleotide polymorphisms (SNPs).
- K/k: 698 T→C (changes methionine to threonine at a.a. 193)
- Duffy T→C in the GATA transcription initiation sequence prevents binding of the GATA transcription factor and no transcription occurs; the patient will be Fy(a-b-)
- Finally, there are other genetic polymorphisms that can affect red blood cell antigens
  - Most Rh negatives (D negative) result from a deletion of the entire “D” gene
  - Recombination events affect the MNS and Rh systems
  - Insertion of a nucleotide: Colton blood group and Rh

Molecular Testing: Methodology

Outline of Steps

1. DNA Isolation
2. PCR Amplification
3. Post-PCR Processing
4. Detection
Molecular Testing: Methodology

Detection

• Three methods have been used:
  • Gel-based (Low resolution; Medium throughput)
    • Earliest tests utilized PCR to amplify specific regions of the target blood group gene
    • Primers designed to amplify region of blood group gene known to contain the SNP causing different antigens to be expressed
    • PCR products digested with "restriction enzyme"
    • DNA products separated on agarose gel: "restriction fragment length polymorphism (RFLP)"
  • Sequence-based (High resolution; Low throughput)
    • Two general methods have been used; currently a modification of the "Sanger" chain termination method is preferred
  • Bioarray Technologies (Medium resolution; High throughput)
    • Use either glass slides dotted with DNA probes while others are bead-based technologies
    • A "Bioarray" technology has been licensed by the FDA for blood group antigen testing

Molecular Testing Steps:

Summary

DNA isolation

Healthcare

Chromatography

Analysis

Detection

Interpretation
Molecular Testing: What is this good for?

- Transfusion Service:
  - Perinatal testing
    - RHD type to predict need for RhIg
    - Genotyping fetal DNA to predict risk of HDFN
  - Providing genotyped matched blood
    - Patients with SCD
    - Patients with thalassemia
  - Chronically transfused patients
  - Genotyping patients
  - Recently transfused patients
  - Patients with autoantibodies
  - D-typing of patient to predict need for RhIg or D-negative blood products

Perinatal Testing

- Perinatal Testing
  - RHD Typing for Rh Immune Globulin
  - Genotyping fetal DNA to predict HDFN risk

To understand the impact of molecular testing and the implications of recent recommendations, we have to spend a few minutes describing the Rh gene locus:

- The Rh blood group system is perhaps the most complex and is made up of 54 serologically identified antigens
- These antigens are encoded by 2 homologous, closely linked genes on chromosome 1
- These genes encode complex, hydrophobic proteins that span the red cell membrane 12 times:
Perinatal Testing

- Rh Genes (Continued):
  - The RHD gene is responsible for encoding the RhD antigen; while the RHCE gene encodes the C/c and E/e antigens.
  - These two genes are each composed of 10 exons and are 93.8% homologous.
  - Have opposite 5' → 3' orientations.
  - Crossing over between RHD and RHCE genes as well as a variety of other genetic changes are responsible for the many genomic recombinations seen in the Rh system.

- RHD Gene
  - With respect to "perinatal testing" we will now focus on the RHD gene.

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RHD

- The complexity of D antigen expression has led to confusion and inconsistency in transfusion medicine:
  - CAP 2014 Survey (3100 laboratories):
    - When testing for serologic weak D, 47% report patient as D+; 11% report patient as D-; and 50% just use the term "weak D".
    - 20% of laboratories perform serologic weak D testing on all patients while 10% perform this testing only on pregnant women or women of child-bearing age.
  - This issue is magnified when decisions about the use of Rh immune globulin to prevent anti-D formation and HDFN.
    - 50% of these institutions would NOT give RhIg to pregnant women found to be weak-D delivering an Rh+ child.

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The RHD Gene

- Let's descend a little deeper into the depths of the Rhesus genome!!
  - The RBCs of D negative individuals lack the RhD polypeptide in their RBC membrane.
  - There are 3 ways in which the Rh negative phenotype can occur:
    - A complete deletion of the RHD gene. Most Caucasians; 19% of people of African ethnicity.
    - RHD-C-E hybrid; expresses no D antigen. 15% of D- negative people of African ethnicity.
The RH D Gene

- The difference between Rh positive and Rh negative is then pretty simple:
  - Rh positive: you have the RH D gene and produce the D antigen
  - Rh negative: you do NOT have a functional RH D gene (one of 3 known mechanisms) and do NOT produce the D antigen

- Unfortunately, for those who have a functional D gene, there are over 100 variants that can be divided into 3 groups:

Classifying RH D Variants

Weak D’s
- 76 different weak D’s that cause changes in the reduced expression of the antigen (low copy number)
- React ≤2+ with anti-D reagent
- Can be managed as Rh positive for transfusion and pregnancy
- Do NOT produce anti-D when exposed to Rh positive blood

Del
- 10 different Del allelic
- Low expression of D due to severely reduced expression of the antigen
- Can stimulate anti-D in D neg patients

Partial D’s
- Known SNPs that alter D expression generally external to membrane
- 45 different hybrid genes
- Type as D pos or D neg (Some Partial Ds react strongly)
- Can not be distinguished serologically
- Can produce anti-D when exposed to Rh positive blood

RHD Variants and Molecular Testing

- Molecular testing is able to distinguish among these D antigens variants while traditional serologic testing cannot:
Weak D and Alloimmunization

• Perhaps most importantly, there is a correlation between these variants and the risk of alloimmunization (anti-D) when individuals with one of these variant D antigen types is exposed to Rh positive blood through transfusion and pregnancy:
  • Weak D types 1, 2, and 3
    • Most common weak D variants in Caucasian population (up to 90% of weak-D)
    • Not at risk for producing anti-D when exposed to Rh positive blood through transfusion or pregnancy
    • Not candidates for Rh immune globulin
  • Partial D
    • Relatively more common in African Americans (>25%)
    • Patients with partial D at risk of producing anti-D when exposed to Rh positive blood
    • Candidate for Rh immune globulin

Case Study

• History:
  • A 24 year-old Caucasian G2P1 female is 12 weeks pregnant. She types as A positive and has a negative antibody screen. However, she was typed as A negative at the time of her previous pregnancy when she received Rh immune globulin at 28 weeks of gestation and after the delivery of her Rh positive infant. Due to this Rh testing discrepancy, molecular testing is performed

• Results:
  
<table>
<thead>
<tr>
<th>Allele 1</th>
<th>Allele 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD*01W.01 (Weak D Type 1)</td>
<td>RHD*N01 (Does not express RhD)</td>
</tr>
</tbody>
</table>

Summary: Predicted phenotype Weak D+; This patient's D antigen is weak and may be detected by only some and D reagents which accounts for the typing discrepancy between her 2 pregnancies. Rh immune globulin is NOT indicated
The Practical Implications

• Recommendations:
  • RHD genotyping should be performed whenever a serological weak D phenotype is detected
  • Weak D Types 1, 2, and 3 should be managed as RhD-positive with regard to administration of Rh immune globulin or for transfusion
  • For women with a serological weak D phenotype associated with an RHD genotype other than weak D type 1, 2, or 3, the Workgroup recommends conventional prophylaxis with Rh immune globulin

The Potential Benefits of RHD Genotyping:

Pregnant Women and Unnecessary RhIg Injections
Financial Implications

- Cost-benefit analysis:
  - Goal: evaluate costs of RHD genotyping for pregnant females with serologic weak D phenotypes
  - Comparison strategy of managing women as D negative
  - Costs assessed over 10 and 20 year periods
  - RHD genotyping is cost-saving over treating as Rh negative when genotyping is ~$256

The Potential Benefits of RHD Genotyping:

Unnecessary Rh negative Blood Use

- Providing blood for chronically transfused patients
- Sickle cell disease
- Thalassemia
Transfusion in Sickle Cell Disease

- RBC transfusion is a mainstay of therapy for patients with sickle cell disease (SCD).

<table>
<thead>
<tr>
<th>Name</th>
<th>Classic TxRx (allergic, febrile, etc)</th>
<th>Iron overload</th>
<th>Alloimmunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
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</tr>
</tbody>
</table>

- Just as transfusion is used to treat many of the complications of SCD, transfusion, itself, presents its own set of complications:
  - Classic TxRx (allergic, febrile, etc)
  - Iron overload
  - Alloimmunization
    - 20-30% SCD develop RBC antibodies in up to 5% of patients with thalassemia
    - Delayed hemolytic transfusion reactions (5-20% of patients)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>SCD Patients (%)</th>
<th>Donor (%)</th>
<th>P Value</th>
<th>Antibody Frequency (%)</th>
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</thead>
<tbody>
<tr>
<td>c</td>
<td>98</td>
<td>99</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>e</td>
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<td>s</td>
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</tr>
<tr>
<td>f</td>
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<td>88</td>
<td>&lt;0.001</td>
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</tr>
<tr>
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<td>&lt;0.001</td>
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</tr>
<tr>
<td>Kell</td>
<td>91</td>
<td>88</td>
<td>&lt;0.001</td>
<td>10</td>
</tr>
</tbody>
</table>

- Reasons for Alloimmunization in SCD Patients:
  - Number and frequency of transfusions seems unrelated to the frequency of alloimmunization in SCD
  - Disparity in RBC phenotypes between donors and patients might play a more important role:
Transfusion in Sickle Cell Disease

- Minimizing the Risk of Alloimmunization in SCD:
  - Phenotype matching
  - In 2 recent studies the genetic diversity and presence of blood group variants demonstrate the potential benefit of genotyping (rather than phenotyping) these patients……..


O’Suoji et al (2013)
- 641 SCD pediatric patients
- 180 transfused with RBCs screened for C, E, and Kell antigens
- 26 patients became alloimmunized; 16 of which developed anti-C, anti-E or anti-Kell
- All but 5 of the patients who developed C, E, or Kell antibodies had been transfused at institutions where extended phenotyping was not practiced
- The remaining 5 patients were shown by phenotyping to have variant genes

Chou et al (2013)
- 182 patients with SCD
- RBCs screened for C, E, and Kell antigens
- 62 patients alloimmunized to Rh antigens (146 antibodies identified; 91 unexplained Rh antibodies)
- 56 of the unexplained Rh antibodies occurred in patients who were phenotypically positive for the antigen
- Genotyping revealed that 87% of these patients had variant alleles

Transfusion in Sickle Cell Disease

- Commercial blood typing reagents detect the expression of only 5 Rh antigens: D, C, c, E, and e
- There are over 200 variants of RHD and 80 known variants of RHCE, and these variants can be identified by molecular testing but cannot be distinguished by routine serologic testing
- RHD and RHCE variants are found in <1-2% of Caucasians, the frequency is much higher in individuals of African descent
- In a cohort of 320 patients with SCD, 27-40% demonstrate variant genotypes—many of which can result the antibody formation
Patient Genotyping

- Patients with autoantibodies
- Patients who have been recently transfused

Patients with Autoantibodies

- Patients with warm-reacting autoantibodies are a difficult transfusion challenge:
  - Many patients with warm-reacting autoantibodies require transfusion
  - However, due to the presence of IgG autoantibodies in the patient serum, all red blood cell units will be incompatible on crossmatch and our ability to type the RBCs is limited to the presence of IgG on the surface of the red cells (Positive DAT)
  - Even though red blood cell units transfused to a patient with warm autoantibodies are usually well tolerated, the real concern is the fairly common presence of alloantibodies in these patients masked by the autoantibody—28% - 40% of patients with autoantibodies also had alloantibodies.
  - Detection of these "masked" alloantibodies requires time-consuming absorption and elution techniques often not routinely available in many transfusion services
  - So, how can we effectively manage this situation?

Patients with Autoantibodies

- One approach to the management of these patients is the determination of an extended red blood cell phenotype:
  - Having knowledge of the patient’s phenotype facilitates the interpretation of serum reactivity since patients will develop alloantibodies only to antigens that they lack
  - This is particularly useful if transfusion is urgent; in this situation RBC units can be selected lacking antigens to which the patient can develop antibodies
  - While screening units to eliminate those with specific antigens may seem time-consuming, it is certainly more quickly accomplished than the labor-intensive adsorption and elution procedures or reticulocyte isolation procedures that would otherwise be necessary
  - In a 2002 publication in Transfusion from Johns Hopkins, it is recommended that "complete phenotypes should be a routine component" in patients with autoantibodies but this procedure circumvents or simplifies pre-transfusion adsorption studies
Patients with Autoantibodies—Molecular Testing

- Molecular testing provides another approach to red cell phenotyping in patients with autoantibodies.
  - Illustrative case study:
    - 78 year-old male with CLL and autoimmune hemolytic anemia. Received RBC transfusion every 2 weeks. Known to have a history of anti-C, anti-Kell, and anti-Kpa. Prior to each transfusion, absorption and elution techniques were required to rule out new alloantibodies. No pre-transfusion serologic phenotype was available. Molecular testing was performed and he was found to be positive for most common clinically significant antigens, but negative for C, Kell, Kpa, and Fyb. All subsequent RBC units for transfusion were selected to be negative for C, Kell, Kpa, and Fyb. No adverse reactions occurred during future transfusions.

Clinical Uses of Molecular Testing

- The potential uses of molecular testing (genotyping) of patients are outlined in an article from the Cleveland Clinic and include:
  - Patients requiring phenotype-matched blood (e.g. Sickle cell disease)
  - Patients with autoantibodies or other serologic reactions that prevents exclusion of clinically significant antibodies (Multiple antibodies, HTLA)
  - Patients with suspected antibodies for which typing antisera are not available (e.g. K, Jka)
  - Patients with discrepancies in serologic test results

Propylactic Phenotype Matching: Algorithm
Summary

- Current blood bank testing
  - Serology
  - Procedures
- Molecular testing
  - What is this and how does it work?

Clinical uses of molecular testing

- Prenatal testing
  - Fetal DNA
  - DNA for karyotyping
  - DNA for RHD zygosity
- RHD‐weak‐D and Partial‐D (Rh immune globulin treatment)
- NAIT
  - Providing phenotype‐matched RBC units
- Sickle cell disease
  - Beta thalassemia
- Patient genotyping
- Recently transfused patients
- Patients with previous alloimmunization
- Patients with unexplained reactions

Any Questions???????????????????