ABO isoagglutinin titration: application, method and controversies

Dr Ashish Jain,
Associate Professor,
Department of Transfusion Medicine, PGIMER, Chandigarh
**ABO isoagglutinins**

- **Isohemagglutinins** are naturally present antibodies to non-self A and B blood group antigens.
- Also k/a **ABO hemagglutinins** or **ABO isohemagglutinins** or **ABO antibodies**.
- Occur as ‘naturally occurring’ antibodies; but their origin is still unclear.
- Whether these antibodies are produced through some inherited, “natural” innate mechanism, not requiring antigenic stimulation or, instead, follow classical adaptive immune-mediated mechanisms.

---

**Landsteiner’s Law**

Whichever ABO antigens are lacking on a given person’s RBCs, that person will *always* have the corresponding antibody or isohemagglutinin.
Development of ABO isoagglutinins

- In most infants, anti-A and anti-B agglutinins (presumably IgM) produced by the infant can first be demonstrated at 3–6 months.
- The titre of anti-A and anti-B agglutinins reaches its maximum at the age of 5–10 years.
- It may be wholly IgM or partly IgM and partly IgG, partly IgM and partly IgA or may be made of all three immunoglobulins.
Characteristics of ABO isoagglutinins

- IgG anti-A and anti-B are found far more commonly in group O than in B or A subjects.

- **IgG subclasses:** $\text{IgG}_2 \geq \text{IgG}_1 \geq \text{IgG}_3$

- Both IgM and IgG may be hemolytic, bind complement; IgA is not hemolytic.

- Naturally occurring anti-A and anti-B react more strongly at 4°C than at 37°C.

- **Cross reacting anti-A,B:** Anti-A,B in group O serum is an antibody directed against an epitope shared by both A and B (cannot be distinguished by differential adsorption).
Applications of ABO Ab titration

- Hemolytic disease of the fetus and newborn (HDFN): IgG anti-A, anti-B, and anti-A,B are all capable of causing HDFN: almost only occurs in \( A_1 \), \( B \), or \( A_1B \) babies of group O mothers.

- ABO incompatible solid organ transplantation: ABO antibodies can cause hyperacute rejection of incompatible kidney, liver, and heart transplants.

- ABO incompatible Hematopoietic stem cell transplant (HSCT): HSC do not express ABO antigens, so ABO is often disregarded when selecting a stem cell donor. However, major ABO incompatibility may lead to hemolysis of infused red cells with a bone marrow transplant.

- Transfusion of platelets containing ABO incompatible plasma: screening for donor anti-A and anti-B hemolysins, and high titers of IgM and IgG is suggested when using ABO non-identical platelets.
Titration is a semi-quantitative technique of measuring the concentration of an antibody in a serum. It is performed using the double dilution technique (Serial dilution). Dilution is expressed as 1 in 16, which means the dilution factor is 16. Titer is simply the inverse of dilution at which the endpoint agglutination (1+) is achieved.
RBC phenotype: A1 or A2

Concentration of RBC in final mixture

Factors affecting ABO Ab titers

Time and temperature of incubation

Technique of reading the endpoint

Wider range for anti-A (8-2048) than anti-B (8-256)

Redman et al (UK, 1990) showed that there is no significant difference between Black, White and Asian people
**Tube technique (TT)**

- It is the most commonly performed method in laboratories.
- The room temperature (RT) incubation technique and the indirect antiglobulin test (IAT) have been interpreted as the methods detecting IgM and IgG, respectively.
- Both IgM and IgG of ABO Ab can agglutinate RBCs at RT (20-24°C) or below and efficiently activate the complement at 37°C.
- Therefore titers using RT techniques or IAT on dithiothreitol (DTT) untreated samples may be more reflective of the mixed concentration of IgM and IgG of ABO Ab.
Complete and Incomplete antibodies

Complete Antibody (IgM)

Incomplete Antibody (IgG)

Add antihuman globulin serum
Mix and centrifuge
Read and interpret the agglutination reactions
Titer: doubling dilution

Label 10 test tubes (1:1, 1:2, 1:4, 1:8, 1:16, 1:32............)

Add one volume (100µl) of saline to all test tubes except the first tube

Add an equal amount of serum to each of the first two tubes

Using a clean pipette mix the contents of the 1 in 2 dilution several times and transfer one volume (100µl) into the next tube

Continue the same process for all the dilutions, using a clean pipette to mix and transfer each dilution and save the last transfer volume

Add 1 drop of the corresponding red cell suspension (5%) into each test tube. Mix well, keep these test tubes at room temperature for at least 15min, centrifuge at 1000 rpm for 1min

Observe the highest dilution that produces macroscopic agglutination (1+)
Titer: doubling dilution (contd...)

Normal saline

100μl

Serum
NEAT
## Tube Agglutination Grading

<table>
<thead>
<tr>
<th>Scale</th>
<th>0-4</th>
<th>0-12</th>
<th>Macrophocically Observed Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12</td>
<td></td>
<td>One solid agglutinate, no free red cells detected.</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td></td>
<td>One or two large agglutinates, a strong reaction.</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td></td>
<td>Medium size agglutinates, clear background.</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td></td>
<td>Small agglutinates, with a lot of free red cells.</td>
</tr>
<tr>
<td>+/-</td>
<td>3</td>
<td></td>
<td>Weak granularity in the red cell suspension.</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td></td>
<td>No agglutinates, an even red cell suspension.</td>
</tr>
</tbody>
</table>
Disadvantages

- Variation in cell suspension
- Cell loss during washing
- Alteration in cell:serum ratio
- Inter-observer variation (1+ and wk+ are subjective: End-point ??)
- Stability of test results
- Variation in repeat testing
Column agglutination technology (gel)

- Conceptualized by Lapierre (1985)
- Principle: Controlled centrifugation of red cell with/without serum through a porous dextran or polyacrylamide gel column of defined pore size under defined sets of incubation.
- Gel acts as a sieve so that unagglutinated cells settle at bottom and cells forming lattice get trapped at various zones across the column.
- LISS/COOMBS gel card contains Anti-IgG(Poly) + Anti C3d (mono)

- Preparation of 0.8%-1% LISS suspension of red cells (No need to wash the cells)
- Dispense 50µL in the reaction chamber at acute angle
- Dispense 25µL of recipient serum on its top gently
- Incubate for 15 minutes at 37*C
- Spin at 1000 rpm for 10 minutes
- Interpret
Advantages

- more qualitative in grading the strength of agglutination reaction
- the inter-observer variation is minimal
- less time-consuming
- uses smaller volumes of serum and RBCs

Limitation: COST ??
Microplate technique

**MICROPLATES**
- Small tray with 96 small wells
- Holds 200-300 microlitres of reagent
- Three types: V-type, flat-bottom, U-type

**Advantage**
- More *sensitive* — very weak cell suspension can be used
- Very *small* amount of reagents are needed
- Titrations are *easier* with multi channel pipettes
- Grades of reaction can be *compared*

**Disadvantage:** high viscosity in serum/plasma causes red cells to adhere to side of wells
Solid phase red cell adherence assay (SPRCA)

- Components of antigen-antibody reaction is immobilized onto a solid medium.
- On centrifugation antigen positive cells spread out while antigen negative cells form a button at the bottom of the well.
- Excess plasma is blotted out and anti IgG bound indicator red cells are added to give visible reaction.

SPRCA: Available in automated platforms
**Indirect test**

1. **Antigen coat**
2. + Test serum or plasma, incubation at 37°C
3. **Antibody attached to RBC’s antigen**
4. Wash to remove unbound antibody
5. + Indicator RBC
6. **Negative**
7. **Positive**
Flow cytometry

- **FLOW**: In motion.
- **CYTO**: Pertaining to cells.
- **METRY**: Measurement.

- It is a technology that measures various properties of cells/particles of interest in a sample based on markers present by passing them in a fluid stream under a beam of light.

- It deals with,

  IDENTIFICATION
  ANALYSIS
  PURIFICATION
  (SORTING)
  PHYSICO-CHEMICAL
  PROPERTIES

- Any particle that can be suspended in a fluid: cells, chromosomes, and individual molecules, can be characterized by flow cytometry.
Detection of ABO antibodies by flow cytometry

- **ABO fluorescence-activated cell sorting (ABO-FACS)** to quantify binding of anti-A/B IgM, IgG and IgG subclasses to human A or B red blood cells.
- The sensitivity and specificity of anti-A/B IgM to predict the blood group was 93% and 96% respectively.
- IgG2 was the predominant IgG subclass.
- The correlation of anti-A/B IgM and IgG in the ABO-FACS with haemagglutination titres was 0.870 and 0.783, respectively (n= 240; P < 0.001).
- It opens the possibility of isotype-specific monitoring of anti-A/B antibodies levels after ABO-incompatible solid organ and stem cell transplantation.

Controversies in ABO titration

- The preferred method?
- Standardization?
- End points?
- Critical titre levels (specially for ABOi-KT)?
Comparing the Tube and Gel Techniques for ABO Antibody Titration, as Performed in Three European Centers

Data from 60 consecutive ABO-incompatible kidney transplantations performed in Stockholm, Sweden; Freiburg, Germany; and Uppsala, Sweden, revealed significant variation in preoperative A/B antibody levels, with median titers of 1:32, 1:128, and 1:8, respectively. We wanted to investigate whether these differences were method-related. The same samples from 21 healthy blood donors were analyzed in the three centers using current local methods. Results confirmed method-related differences, with higher A/B titers in Freiburg and lower titers in Uppsala compared with Stockholm. Results for the same sample differed by a median of three (range 0 to 6) titer steps. When the same number of samples were analyzed in the three centers using the same gel method and the same test erythrocytes, results differed by a median of one titer step (range 0 to 4) for the same sample. In conclusion, gel hemagglutination technique significantly decreases intercenter variation compared with tube technique.

(Transplantation 2007;84: S17–S19)
The AHG titer results using the Tube Technique (TT) method were compared to IgG gel titers. Forty-three (86%) of the titer results were identical and 7 (14%) varied by one standard dilution. Five (10%) were one dilution greater than the TT-AHG titer, and two (4%) were one dilution lower than the TT AHG titer. No IgG gel titer varied more than one standard dilution from the TT AHG titers.

CONCLUSIONS: The Tube and IgG gel titers are comparable. The IgG gel method offers the best titer turnaround time, eliminating 45 minutes of incubation time alone. Implementation of this technique would benefit ABO INKT patients by providing titer results in a more timely manner.
• **CTT titration method:** Titrations were incubated at ambient (22-25°C) RT followed by incubation for 30 minutes at 37°C with subsequent conversion to the AHG test phase using monospecific anti-IgG.

• **Revised TT titration method:** Titrations were performed according to the CTT titration method except the 30-minute RT incubation test phase was omitted.

• **Anti-IgG gel titers:** Titrations were performed using anti-IgG gel cards (Micro Typing Systems, MTS, Ortho Clinical Diagnostics, Raritan, NJ)

Table 3. Comparison of CTT AHG titers that included a RT test phase to the titers determined using anti-IgG gel cards

<table>
<thead>
<tr>
<th>CTT AHG* titers</th>
<th>Number</th>
<th>Identical to CTT AHG titer</th>
<th>&gt;CTT AHG titer</th>
<th>&lt;CTT AHG titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>18</td>
<td>13</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>32</td>
<td>7</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>64</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>256</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>26 (52%)</td>
<td>12 (24%)</td>
<td>12 (24%)</td>
</tr>
</tbody>
</table>

* CTT AHG titers = titrations that included a 30-minute room incubation phase at RT.
† IgG gel titers = titrations performed using anti-IgG micro-column gel method.

Table 4. Comparison of revised TT AHG titer results with the IgG gel titers

<table>
<thead>
<tr>
<th>Revised TT AHG titer*</th>
<th>Number</th>
<th>Identical to revised TT AHG titer</th>
<th>&gt;Revised TT AHG titer</th>
<th>&lt;Revised TT AHG titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>18</td>
<td>16</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>7</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>64</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>6</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>256</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>43 (86%)</td>
<td>5 (10%)</td>
<td>2 (4%)</td>
</tr>
</tbody>
</table>

* Revised TT AHG titer = titers determined using a revised TT method without a RT incubation phase.
† IgG gel titer = titration using anti-IgG micro-column gel method.

Streamlining ABO titration

- The IgG gel method offers the best turnaround time requiring only 15 minutes of incubation at 37°C and eliminates the tedious reading of TT agglutination reactions.
- The gel reactions are stable, batch titrations can be easily accommodated by IgG gel.
- AuBuchon and co-workers have reported that using a weak-positive titer end-point may reduce titration variability.
End-points

- The variance between laboratories was not significantly reduced with the uniform method using a 1+ end-point.
- A statistically significant reduction in the variance of anti-D and anti-A titres by the TT (including the IAT phase) was seen when 19 laboratories re-analysed their results using a w+ end-point.
- Titration against red cells of the specified phenotype provided by the participating laboratory did not appear to introduce additional variance.
- Results reported based on the gel card technique at the AHG phase (1+ end-point) showed reduced variance compared to tube-based techniques.

SPRCA v/s Gel

- ABO titration assays on the Fully automated SPRCA platform (Galileo-NEO, Immucor).
- 318 IgG and 105 IgM titrations were performed.
- The results were compared to the manual gel card method (Bio-Rad) without pretreatment with DTT.
- The typically one dilution difference vs the gel card method can be possibly explained by the presence of IgM affecting the gel card and that different antigen concentration are employed to that in the automated assay.

**Results:** Titration assays show good reproducibility when lots of reagents are kept constant and specimens are run on the same instrument (endpoint titer the same or maximally changed by one dilution). Moreover, reagent lot to lot studies and instrument to instrument studies demonstrated good reproducibility (endpoint titers maximally changed by 2 dilutions). The system throughput is 12 specimens in 30 min.

Median titers of anti-B and anti-A in all blood groups were higher in CAT without DTT than in CAT with DTT, especially for group O individuals.

Conclusions: We recommend CAT with and without DTT for titration of anti-A and anti-B, especially in group O individuals, to provide more sensitive results that include IgG data.

Park ES et al. Comparison of total and IgG ABO antibody titers in healthy individuals by using tube and column agglutination techniques. Ann Lab Med 2014;34:223-229
Case (PGIMER, Chandigarh)

- Patient NK; 31yr/ Male; CKD ESRD
- Posted for ABO incompatible renal transplant.
- Blood group of the patient: O RhD Positive
- Donor: A₂RhD Positive
- Antibody screen and DAT: Negative (Gel)
- Titers:

<table>
<thead>
<tr>
<th>Anti-A Titer</th>
<th>TUBE TECHNIQUE</th>
<th>GEL TECHNIQUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ig M</td>
<td>Ig G</td>
</tr>
<tr>
<td>Pre-TPE-1</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Post-TPE-1</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Desensitization: Glycosorb
Serial titers (IgG Gel)

<table>
<thead>
<tr>
<th>Pre-TPE</th>
<th>Post-TPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre TPE</td>
<td>Post TPE</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-2</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-6</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-7</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-8</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-9</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-10</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-11</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-12</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-13</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-14</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-15</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-16</td>
</tr>
<tr>
<td>Post Renal Tx</td>
<td>Day-17</td>
</tr>
</tbody>
</table>

Ig M

Ig G

GLYCOSORB
Conclusion

- Interlaboratory variations in the technical procedures and results do occur in measurement of the ABO Ab titer.
- CAT significantly decreases variation as compared to the tube test.
- Individual centres should develop their own protocols based on:
  - Available resources
  - Validation of applied methods
  - Goals of isoagglutinin titer
- A periodically conducted assessment could help in continued improvement of the results of ABO Ab titer measurement.
Thank You