DETECTION AND IDENTIFICATION OF ANTIBODIES

SUMMARY
Antibody Screen:
- Detect Unexpected Antibodies (0.2 – 2%)

Unexpected Antibodies
a) Immune = RBC stimulation in the patient
b) Passive = transferred to the patient through blood products/derivatives
c) Naturally occurring = environmental factors

Autoantibodies
- Foreign antigen

Clinically Significant Antibodies: shorten RBC survival and target antigen.
- IgG that reacts in 37°C or AHG phase = HTR, HDFN

Screen cells = commercially prepared from group O RBC suspension from donors

Homogenous individual
RBC = double dosage in a single antigen
→ Because of inheritance of 2 genes that code for the same antigen

Heterogenous individual
RBC = single dosage in each single antigen.
→ Each gene codes for a different antigen

DOSAGE: (yield stronger reaction against RBC with homoyzous expression)
- Kidd (Jk\(^a\), Jk\(^b\))
- Duffy (Fy\(^a\), Fy\(^b\))
- Lutheran (Lu\(^a\), Lu\(^b\))
- Rh (C, Ee)
- MN
- Ss

Enhancement reagents:
→ Added to serum & cell mixtures in IAT = promote Ag-Ab binding (Agglutination)
- LISS
- Saline
- Albumin
- PEG (no 37C phase)

Coomb’s Control Cells = RBC coated with human IgG
→ Added to all AHG (−) tube test
→ Ensure adequate washing
→ Ensure AHG reagent is present and functional

Antibody Exclusion
- Rules out possible antibodies based on antigens that are present on negative reacting cells.

Conclusive Antibody Identification
- Serum (w/Antibody) is reactive with ≥3 Antigen (+) cells
- Serum (w/Antibody) is non-reactive with ≥3 Antigen (+) cells
- Patient RBC phenotype negative for the corresponding antigen.

DAT
- Detects RBC sensitized with Ab in vitro

Elusion methods
- To free antibody from cell surface
- To allow identification

Calculation
- Determine if random donor units that should be Antigen-typed
- To provide the requested #Ag (−) RBC units for patient with an antibody
- Divide #Ag (−) units by frequency of Ag (−) individuals in donor population

Relative quantity of RBC antibody
- Determined by testing serial 2fold dilutions of serum against Ag (+) RBC
- Reciprocal of highest serum dilution showing agglutination = Ab titer

PURPOSE, TO INVESTIGATE:
- HTR
- Immune Hemolytic Anemia
- HDFN

UNEXPECTED ANTIBODIES:
1) Immune Alloantibodies
- Because of RBC stimulation through:
  - Transfusion, transplantation, pregnancy
2) Naturally occurring Antibodies
- Because of exposure to environmental sources:
  - Fungus, pollen, bacteria (have similar structures to some RBC antigens)
3) Passive Acquired Antibodies:
- Antibodies produced in 1 individual
- Transmitted to another individual
- Via Plasma-Containing blood components or
- Via derivatives (IVIG)

CLINICALLY SIGNIFICANT ANTIBODIES
→ Cause decreased survival of RBC processing the target antigen

Usually:
- IgG that reacts at 37°C or AHG/IAT phase.

Autoantibodies
- Directed against self antigen
- May mask clinically significant alloantibodies

Antibody Detection/ Screen → Antibody Identification/ Panel
Antibody’s presence (YES/NO) → Which antibody?

ANTIBODY SCREEN
- 0.2 – 2% has detectable RBC antibodies.
- Donor (Ag-typing sera & Ag (−) RBC units)

Required for:
- Pretransfusion compatibility testing
- Evaluation of the compatibility of hematopoietic progenitor cell (HPC)
- Evaluation of the compatibility of bone marrow donors with the recipient
- Standard prenatal testing for obstetric patient:
  - Evaluation of the risk of HDFN (in fetus)
  - Assess mother’s candidacy for RHIG prophylaxis

IF (YES) DETECTED
- Reduce plasma in the product

TUBE METHOD - traditional (IAT)
□ RBC reagents
□ Enhancement reagents
□ AHG reagents

Patient’s serum/plasma + Known RBCs
a) Immediate Spin phase = detect Ab reacting at RT
  - Not required
  - May lead to detection of clinically insignificant cold antibodies
b) 37°C incubation phase
  - IgG sensitize any RBCs that possess the target antigen
  - IgG coat the antigen with antibody
  - Add enhancement media to increase the degree of sensitization
  - Centrifuge, observe for agglutination
c) AHG phase
  - Wash 3x with 0.9% saline to remove all unbound antibodies
  - Add AHG reagent / Coomb’s serum
  - Centrifuge, observe for agglutination ∗Hemolysis = loss of cell button mass
d) If negative, add Check Cells

RBC REAGENTS
- for Antibody screen
  - from O individual (so that Anti-A and Anti-B will not interfere in the detection of antibodies)
  - RBC suspension (4-5%): maintains integrity of antigens, prevents hemolysis
  - Screen cells = packed in 2/3 sets cell suspension
  - there should be 1 cell (−) for each of the antigens (D,C,c,E,e,K,k,Fya,Fyb,Jka,Jkb,Lea,Leb,P1,M,N,S,s)
  - lot specific, shouldn’t be interchanged
- Homozygous expression of antigen = detect antibodies that shows dosage
  - from a person who inherited only 1 allele at a given genetic locus
  - cell surface has double dosage
- Heterozygous antigen
  - from a person who inherited 2 different alleles at a locus
  - alleles share available antigen sites on the cell surface
- Dosage: antibodies that react more strongly with cells having homzygous antigen expression
- Pooled screening reagent: acceptable (from at least 2 different individuals)
  - observe for mf ∗Target antigen may be expressen on only 1 cell of the pool
Avoid time-consuming investigation of insignificant antibodies

Commercially prepared screen cells: detect antibodies and ensure compatibility with donor unit.
SCREEN CELLS = test for clinically significant antigen
CROSS MATCH = possess only some of the antigens
SCREEN CELLS = have cells with homozygous expression, reliable in detecting weakly reacting antibodies that are undetected by crossmatch.

OLDER RBC, WEAKEN Ag expression → dilute commercially prep cells in a PRSV to maintain integrity of antigen.

Advantage of TUBE’S TEST:
- Commonly available lab. equipment
- Relative low cost
Disadvantage of TUBE’S TEST:
- Instability of the reactions
- Subjective nature of grading by technologist
- Amount of hands-on time for technologist
- Failure of washing phase

GEL METHOD

- Microtubule + dextran acrylamide gel
- Screen cells + LISS 0.8%
- Patient’s serum/plasma + screen cells → chamber above the gel

- 1 plastic card = upto 6 chamber/gel microtubules (credit card size)
- Incubate card 37C for 15 min → 1 hour → sensitization
- Centrifuge for 10 min (RBCs are forced out of the reaction chamber down into the gel) (IgG + Ab-coated RBCs → Agglutination)
- Agglutinated cells trapped inside gel because of anti-IgG, and because agglutinates are too large to pass through the spaces between gel particles

- IF NO AGGLUTINATION = RBC will form a pellet at the bottom of microtubule

Advantage:
- As sensitive as PEG tube test
- Fewer hands on steps (no washing, no coomb’s control step)
- Reactions are stable up to 24 hours
- Reactions may be captured electronically (standardized grading of reactions)
- Facilitating review by a supervisor
- MF reactions may be more apparent
- Ability to automate many of the pipetting and reading steps
- Increase productivity

Disadvantage:
- Need incubators & centrifuge to accommodate the gel cards

SOLID PHASE ADHERENCE METHOD

- Immucor’s Capture-R
- RBC antigens coat microtiter wells (not being present on intact RBC)

- Patient’s serum/plasma + well in the SC + LISS → incubation 37C
- Wash the wells to remove unbound antibodies
- Add indicator RBC-coated with anti-IgG
- Centrifuge well for several minutes

Disadvantage:
- Sensitization = indicator cells react with Ab bound to Ag coating the microtiter well → diffuse pattern in the well
- No Sensitization = indicator cells form a pellet in the bottom of the well

Advantage:
- Smaller sample size
- Ideal in pediatric setting
- LISS reagent changes color when added to serum/plasma
- Ensures adequate sample is present in test system

Disadvantage:
- Need for careful pipetting when performing manually
- Inadequate volume of indicator cells → weak positive reaction pattern
- Need incubators, washers, centrifuges that can hold microtiter wells

NEW: EMT/ Erythrocyte Magnetogenetized Technology
- Microtiter wells are coated with anti-IgG
- Paramagnetic wells have been adsorbed onto SC by manufacturer

- SC are incubated in microtiter wells along with patient’s plasma 37C, 20 min
- High density liquid separates the SC/plasma mixture from anti-IgG until microtiter plate is placed on a magnetized shaker
- Magnet pulls the SC through high-density liquid

Disadvantage:
- SC sensitized with Ab will react with anti-lgG coating the well → diffuse pattern
- SC uncoated with antibody → pellet at the bottom of the well
- Unbound Ab will remain in a layer above the high-density liquid → eliminate wash step.

INTERPRETATION

(+) Agglutination/ Hemolysis at any stage → Need Ab Identification
1. In what phase(s) did the reaction(s) occur?
- IgG = Room Temp/ lower (Immediate spin)
- IgM = AHG phase

Most commonly encountered Ab:
- Anti-N
- Anti-I
- Anti-P (IgM)
- Anti-Rh,Kell,Kidd,Duffy

AHG REAGENTS
- For agglutinatio/f i n complete antibodies
  - Coomb’s serum: anti-IgG & anti-C, C4 or anti-C3b,C3d
  - Anti-C3d: more desirable reagent, more abundant on RBC surface during complement activation, fewer false (+) reactions
  - Most MedTech use monospecific AHG reagent with anti-IgG only
  - Avoid time-consuming investigation of insignificant antibodies
  - Negative result, add Coomb’s control cells (RH+ RBC coated with anti-D)

- (+) Coomb’s control cells: -adequate washing
- (-) Coomb’s control cells: - repeat antibody screen from the beginning

AHG REAGENTS
- For agglutination incomplete antibodies
  - Coomb’s serum: anti-IgG & anti-C, C4 or anti-C3b,C3d
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  - Negative result, add Coomb’s control cells (RH+ RBC coated with anti-D)
2. Is the autologous control negative of positive?

Autologous control = Px RBC tested against px serum/plasma
(+): Ab screen, (+) AC = Autoantibodies/antibodies to medication
Evaluation of (+) AC = DAT

3. Did more than 1 SC sample react? If so, did they react at the same strength and phase?
> 1 SC (+)
  - Multiple Ab, Single Ab, Px serum contains autoantibody
  - Different phase & strength → Multiple Ab
  + AC (+) Autoantibodies

4. Is hemolysis or MF agglutination present?
In vitro hemolysis → caused by Anti-Le, Anti-Le, Anti-PP, Anti-Vel
MF Agglutination → Associated with Anti-Sd and anti-Lu, anti-Lu

5. Are the cells truly agglutinated or is rouleaux present?
Px serum (altered albumin-globulin ratio/HMW plasma expander) → cause nonspecific aggregation of RBC (Rouleaux)
- all patient serum
- AC, reverse ABO
- doesn’t interfere with AHG phase of testing (px serum is washed away before AHG reagent)
- dispersed by adding 1-3 drops of saline to test tube

LIMITATION
- SC will not detect Ab when Ah titer has dropped below level of sensitivity for screening method employed
- SC cannot detect Ab directed against low-prevalence antigens (not present on any RBC SC set)
- SC may not detect Ab showing dosage (homozygous expression)

PATIENT HISTORY
- Age
- Sex
- Race
- Diagnosis
- Transfusion
- Transfusion
- Pregnancy history
- Medication (IVIV, RHIG, Anti-Lymphocyte Globulin)
  - Anti-A, Anti-B, Anti-
- IV solution
- Naturally occurring antibodies (Anti-M, Anti-Le) = No transfusion, no pregnancy
- (+) AC/ (+) DAT → RBC Autoantibodies ----
  - Infectious disorders
  - Autoimmune disorder
  - Medication
- Recently transfused (past 3 months) –DHTTR
- Ag Typing → Recent transfused (presence of donor RBC in patient’s circulation) → mf reaction

REAGENTS
- Antibody ID Panel = 11 – 20 group O RBCs with various antigen expression
- Ag expression, include homozygous (double dosage)

EXCLUSION
- 1st step
- Rule-out RBC that gave a negative reaction in all phases of testing
  The Ag on these negatively reacting cells probably will not be the Ab’s target
- Rule-out if there’s homogenous expression of the antigen on the cell
  (exclude a weak antibody that is showing dosage)
- Exception: low-prevalence antigen (rarely expressed homogenously)
  K, Kp, Jk, Lu

EVALUATION OF PANEL RESULTS
- Examine the remaining antigens to see if the reactivity pattern matches a pattern of Ag (+) cells (inclusion technique)

1. In what phase(s) and at what strength(s) did the (+) reaction occur?
Do all (+) cells react to the same degree?
  - Strength of reaction:
    - indicate amount of Ab available to participate in the reaction
    - doesn’t indicate the Ab significance
  - Stronger reaction:
    - due to dosage (homozygous Ag expression, stronger than heterozygous Ag expression)
  - Different reaction strength:
    - indicate presence of more than 1 Ab

A cell that possesses more than 1 of target Ag, may react strongly than a cell possessing only 1 of the target Ag
  - Variable expression
    - I, P, Le, Le, Vel, CH/Rg, Sd" antigens are expressed strongly on some RBC
  - Ab of these antigens may react stronger with 1 panel than the others.

2. Do all the (+) cells react at the same phase? Or do any react at different or multiple phases?
  - Reaction at 1 phase & different cells at another phase:
    - Multiple Ab
    - Single Ab showing dosage
      - IgM are usually insignificant (IS, RT/cold)
      - IgG are significant (AHG), (Anti-D, Anti-E, Anti-K = 37C)

4. Are all commonly encountered RBC Ab excluded?
  - Ab to low-prevalence Ag are uncommon (not necessary to pursue additional testing to exclude these specificities)
  - If Commonly encountered Ab is not ruled out, test selected cells that will rule out the presence of the antibody. Perform additional test.

5. Is the autologous control (last row in panel Ag profile) positive or negative?
  - (-) AC = (+) reaction is caused by alloantibody, NOT autoantibody
  - (+) AC = (+) reaction = autoantibody (can mask the presence of alloantibody)

6. Is there sufficient evidence to prove suspected Antibody?
  - Conclusive Ab ID requires test:
    Patient’s serum + enough Ag (+), Ag (-) RBC → ensure reactivity pattern is not a result of chance alone.

3 AND 3 RULES
3 Ag (+) in (+) reaction
3 Ag (-) in (-) reaction

7. Does the patient lack the antigen corresponding to the antibody?
  - Test patient’s RBC for the corresponding antigen
  - Expected!! = ID results are correct
  - Misidentification of the Ab/False (+) typing
  (+) DAT, because of recent transfusion (3 months)

For phenotyping:
  - Elution to remove Ab-coating
  - EDTA/acid glycine and Chloroquine diphosphate → useful in stripping antibody to allow phenotyping
  "Chloroquine diphosphate"* washed RBC ---- incubate + reagent, RT 30min-2hours---- wash----

(-) DAT = OK to phenotype
- Ac glycine/ EDTA (EGA)*
  - Rapid method to remove antibody
  - Kell antigens are denatured (Patient cells cannot be reliably typed for Kell Ag)

**If Coating Ab resist ELUTION, use ABSORPTION METHOD.

RBC ---- in-cube + diluted antiserum ---- centrifuge ---- harvest supernatant ---- test against a cell with heterogeneous Ag expression

(+): for target Ag = Ab will have been absorbed from diluted antiserum
(-): for target Ag, Ab will remain in antiserum, supernatant will be (+) when tested against heterogeneous cell

MF reaction, because of Recent transfusion
- donor cells that stimulated Ab formation react with typing serum
- patient’s AC don’t react

Phenotype:
Reticulocyte Typing
Px RBC --- microHCt tubes --- centrifuge-----
- *Reticulocytes are less dense than mature RBC,Px reticulocytes should be at the top of RBC layer → use Reticulocytes for Ag typing

(+): CC = test with antiserum for Ag typing
(+): CC = Heterozygous antigen expression (antiserum has sensitivity to detect small Qty of antigen)
(-): CC = Lack the target antigen (confirm reactivity with only the target antigen)

Antigen Typing:
- Tube method
- Solid phase adherence
- Gel methods
*Flow cytometry (detect minimum quantities of antigen)
Molecular methods (PCR – examine DNA for single nucleotide polymorphisms that give rise to various RBC antigen)

Advantages of Molecular methods:
- ability to screen large numbers of donors in short time
- no interference from a recent transfusion/ (+) DAT on RBC
- no limitations due to lack of rare antisera
- no limitations due to the expense in purchasing reagents
- reduce the amount of hands-on time required of the medtech.

ADDITIONAL TECHNIQUES FOR RESOLVING AB ID
SELECTED CELL PANELS
- should have minimal overlap in the Ag possessed
- useful when a patient has a known Ab and medtech is attempting to determine if additional Ab are present.

ENZYMES
→ IF multiple Ab are present (to help separate specificities and allow for ID)
  • Ficin (common)
  • Papain
  • Bromelin
  • Trypsin

Enzymes —— modify RBC surface
(removed sialic acid residues) (denature/remove glycoprotein)
Effect: destroy/enhance certain antigen expression.

a) Enzymes maybe utilized in place of Enhancement Media (LISS, PEG)
In a 1 step enzyme test method.
b) Enzymes maybe used to treat the Panel RBC first, then Ab ID panel is performed with treated cells
--not all specificities can be excluded with enzyme panels (because it destroys some antigens)

Compare reactivity of enzyme treated panel with reactivity of the same cells before enzyme treatment.
Observe which cells reacted (+) in the untreated panel, but did not react (or weak) in treated panel
Observe which cells reacted strong in enzyme phase than in untreated panel.

NEUTRALIZATION
- body may have antigenic structures similar to RBC antigens.
→ This substances can be used to NEUTRALIZE Ab in serum
  (help to separate Ab, help to confirm particular Ab’s presence)
  → Useful when multiple Ab is suspected

=================================================================
Px serum --- incubate + neutralizing substance --- allow soluble Ag to bind with Ab
Perform Ab ID Panel with treated serum
• Neutralizing substances inhibit reactions between Ab & Panel RBC
• Use control (saline+serum) to prove the loss of reactivity that is due to neutralization! (not due to dilution of Ab strength by added substance)
==================================================================
ADSORPTION
==================================================================
Serum + target antigen → Ab bind to Ag (Ab is removed from serum)
• Ag-Ab complex (solid ppt) → removed from test system by centrifugation
==================================================================
• Absorbed serum → test against RBC panel for presence of unabsorbed alloAb
• Adsorbent = RBC/another Ag bearing substances

Common reagents:
1) Human platelet concentrate (adsorb Bg-like Ab from serum)
   HLA Ag on platelet + HLA-related Bg-Ab → leave other specificities in serum
2) Rabbit RBC stroma (REST) (performed with some cold AutoAb)
   - Possess I, H, and IH like structures
   Px serum ---- incubate 4C + REST → remove insignificant Ab
   - Possess structure similar to B and Pj Antigens
Reverse grouping and crossmatching with REST-adsorbed serum is not recommended
<table>
<thead>
<tr>
<th>AUTOADSORPTION</th>
<th>- Remove autoantibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMOLOGOUS ADSORPTION</td>
<td>- Patient's RBC isn't available (px is so anemic)</td>
</tr>
<tr>
<td></td>
<td>- RBC phenotyping</td>
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<tr>
<td>DIFFERENTIAL ADSORPTION</td>
<td>- Difficult phenotyping because of DAT +, or recent transfusion</td>
</tr>
<tr>
<td></td>
<td>- 3 aliquots of Px Serum</td>
</tr>
<tr>
<td></td>
<td>- Each aliquot is adsorbed using different cell: R1R1, R2R2, rr</td>
</tr>
<tr>
<td></td>
<td>- 1 must be (-) for K, 1 must be (-) for Jka, 1 must be (-) for Jkb</td>
</tr>
<tr>
<td></td>
<td>- Enzyme treated</td>
</tr>
<tr>
<td></td>
<td>- Ab ID</td>
</tr>
</tbody>
</table>

DAT & ELUTION TECHNIQUE

**Elution** = release, concentrate and purify Ab
**Eluate** = the solution in which antibody is freed into

**Total elution** = antibody is released, antigen is destroyed (for Ab ID)

**Partial Elution** = Antibody is removed, antigen remain intact (prep RBC for phenotyping, for autoadsorption procedure)

Use Chloroquine diphosphate, EGA, ZZAP

<table>
<thead>
<tr>
<th>Temperature dependent method</th>
<th>- heat = remove Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>- Total elution = elution 56 C, lui freeze (-18C)</td>
</tr>
<tr>
<td></td>
<td>- best detecting IgG</td>
</tr>
</tbody>
</table>

**Organic solvent**

- Dichloromethane, xylene, ether
  - organic solvents + lipids in RBC membrane = reduce surface tension, reversal of van der waals force
  - very potent
  - best for detecting non ABO antibodies
  - time consuming
  - chemical pose severe health and safety hazard
  - carcinogenic, flammable
  - most critical step = washing
  - control = last wash should be non reactive (valid)

ANTIBODY TITRATION

- Determine antibody concentration level
- 2 fold serial dilution of serum + RBC suspension
- Antibody titer level = reciprocal of the greatest dilution
- after initial titer, freeze specimen
- Compare new specimen and initial titer specimen
- significant = 4 fold or greater increase in titer

---used for:
- obstetric patient
- pregnancy (HDFN risk)
- intrauterine exchange transfusion
- Rh Ig administration
- detect HTLA antibodies (high titer low avidity)
  = anti-Ch, anti-Rg, anti-Csa, anti-Yka, anti-Kna, anti-McCa, anti-JMH

PROVIDING COMPATIBLE BLOOD PRODUCTS

\[
\begin{align*}
\text{#Units to be typed to find X antigen} &= \frac{\text{# Units requested}}{\text{Frequency of X - individual}} \\
\text{#Units to be typed to find X antigen} &= \frac{\text{# Units requested}}{100 - (\text{Frequency of X+})}
\end{align*}
\]

SUMMARY

- Enhancement reagents = LISS, PEG (added to IAT to promote Ag-Ab binding/agglutination)
- Coomb's CC = RBC coated with human IgG \( \rightarrow \) added to AHG negative test
- to ensure adequate washing, and ensure AHG is present
- Gel & solid phase adherence method = alternative to tube testing
- Antibody exclusion method = rules out possible Ab baed on antigen present on negatively reacting cells
- Conclusive Ab ID = serum containing Ab is reactive with at least 3 Ag+ cells, and negative with at least 3 (-) cells
- DAT = detects RBC sensitized with antibody in vivo
- Elution method = to fee antibody from cell surface for ID