Can Consensus in Single Antigen Testing be Achieved?

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How can you agree that a test result is clinically relevant if you can’t agree on the test result?
Sources of Variability in HLA Antibody Detection - Cellular

Cytotoxicity (CDC)  AHG Enhanced Cytotoxicity  Flow Cytometry

- cell panel composition
- linkage disequilibrium
- number of washes
- autoantibodies
- condition of cells
- effects of drugs*

- same as for CDC but also: source and lot of AHG
- can’t use AHG for B cells

- Fc receptors
- pronase – HLA expression
- serum:cell ratio
- condition of cells
- effects of drugs*
- variability in cytometers, fluorochromes, reagents

*steroids, rituximab, IVIg, ATG, etc.
### Sources of Variability in HLA Antibody Detection – Solid Phase

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Flow Bead Array</th>
<th>Luminex Bead Array</th>
</tr>
</thead>
<tbody>
<tr>
<td>antigen source</td>
<td>epitopes spread across a few beads</td>
<td>epitopes spread across many beads</td>
</tr>
<tr>
<td>antigen density</td>
<td>antigen source</td>
<td>denatured antigen</td>
</tr>
<tr>
<td>Ig isotype and subclass</td>
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<td>interfering factors*</td>
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<tr>
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<td>latex antibodies</td>
</tr>
<tr>
<td>no single antigen for class II – linkage disequilibrium</td>
<td>manual vs automation</td>
<td>antigen source</td>
</tr>
<tr>
<td></td>
<td>number of washes</td>
<td>Ig isotype and subclass</td>
</tr>
<tr>
<td></td>
<td>serum:bead ratio</td>
<td>manual vs automation</td>
</tr>
<tr>
<td></td>
<td>variable antigen density</td>
<td>number of washes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>serum:bead ratio</td>
</tr>
<tr>
<td></td>
<td></td>
<td>variable antigen density on bead</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exaggerated antigen density</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DQA and DPA chain effects</td>
</tr>
</tbody>
</table>

*IgM, complement C1, immune complexes, IVIg
variable antigen density on beads (staining with anti-class I mAb W6/32)

Note the range in fluorescence intensities. Y-axis indicates the MFI values.

• variability bead-to-bead, lot-to-lot
• low expression loci (Cw, DQ, DP) at high density on SAB
• saturation of beads with high titer serum

epitope spread across many beads

Pre-transplant  

<table>
<thead>
<tr>
<th></th>
<th>dMCF(T/B)</th>
<th>dMESF(T/B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 / 20</td>
<td>537 / 548</td>
<td></td>
</tr>
</tbody>
</table>

Bob Bray, Emory
Day 7

Serum date          dMCF(T/B)       dMESF(T/B)
pre-txp             10 / 20         537 / 548
day 7 post-txp     281 / 294       32,389 / 66,439

Bob Bray, Emory
Influence of Test Technique on Sensitization Status of Patients on the Kidney Transplant Waiting List.

tested pretransplant sera of 534 patients using CDC, ELISA and SAB (1λ)

Table 1: Detection of HLA antibodies using different test techniques in patients on the kidney transplant waiting list

<table>
<thead>
<tr>
<th></th>
<th>Positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA</td>
</tr>
<tr>
<td></td>
<td>CDC</td>
</tr>
<tr>
<td>All patients (^1) (n = 534)</td>
<td>28 (5%)</td>
</tr>
<tr>
<td>Without history of immunization (n = 133)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>With history of immunization (n = 286)</td>
<td>22 (8%)</td>
</tr>
</tbody>
</table>

\(^1\)In 115 patients, the information on immunization history was not available. Luminex cutoff MFI ≥ 1000.

antibodies in non-immunized patients:
• 25% >5,000 MFI (up to 14,400)
• some against common antigens (A*2402, B*0801, etc.)
• most not present in SAB assay by other vendor (Lifecodes)
• most not present on PRA bead assay (natural antigens – not recombinant)
Strategies for human leukocyte antigen antibody detection.

8 labs tested one serum by same method, looked at one bead (A*02:01)

Bars indicate the MFI (median fluorescence intensity) reported by each of the eight participating laboratories for the single-antigen bead containing A*0201. Note: although all laboratories agreed on the antigen assignment (HLA- A*02), there was a wide range of values reported.

Inter and intra laboratory concordance of HLA antibody results obtained by single antigen bead based assay.

4 labs used class I and II SAB from a single manufacturer to test 10 patient sera single lot, same SOP, looked at 4 different MFI cutoffs originating lab also tested same samples on four consecutive days.

Standardization of Microparticle-Based, Solid Phase, HLA Antibody Identification Assays

SENDOUT ONE
- 21 Laboratories enrolled (UK, US, France, Germany, Italy) (data from 19 using one vendor)
- Testing performed on 5 well characterized sera using laboratory’s standard methods
- First round of data analysis to identify parameters leading to non-consensus
- SENDOUT TWO
- fewer labs (N=8) using a standardized protocol and same lot of reagents to test 4 sera

- %CV decreases with standard protocol
- Factors NOT correlating with results:
  - Filter vs flick/spin
  - Washes – 3 to 5; Avg: 3.5
  - serum-to-cell ratio
- still differences between labs
- converting to SFI?
- lower MFI – higher CV

16th IHWS Project – Bray and Gebel
Comprehensive Assessment and Standardization of Solid Phase Multiplex-Bead Arrays for the Detection of Antibodies to HLA.

CTOT: Clinical Trials in Organ Transplantation Antibody Core Laboratories
7 labs used 10 kits from 2 manufacturers to test 20 reference sera

- single SOP reduces variation
- still have manufacturer variation
- most variation at low MFI
- MFI cutoff of 1000-1500 had >90% agreement in antigen assignment
- overall CV=25% {so 50% change in MFI (2xCV) means real change in Ab level}

Antibody Consensus Conference – May 2012 recommendations toward standardization

• standardize critical components
  – HLA source and preparation method
  – panel composition
    • appropriate allele coverage, including DQA, DPA, DPB
  – antigen density on bead
  – antigen integrity
    • native vs denatured
  – anti-human Ig detection reagents

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Antibody Consensus Conference – May 2012 recommendations toward standardization

• standardize operating procedure
  – type of plastic trays used
    • V-bottom vs. U-bottom
  – serum volume to bead ratio
  – washing methods
    • spin/flick vs. filter tray
  – vortexing methods
  – unified approach to sample preparation to minimize interference
    • EDTA, DTT, hypotonic dialysis, spin column
  – automated processing equipment

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Antibody Consensus Conference – May 2012 recommendations toward standardization

• standardize interpretation and reporting
  – calibration of fluoroanalyzers and flow cytometers using control particles
  – use of standard fluorescent intensity (SFI) and MESF
  – defined reporting algorithms
  – background normalization
  – reports should include assay type, criteria for positive/negative results, Ig isotype, serum modification, factors that affect test values and interpretation

Tait, et al. Transplantation 2013;95:19
Antibody Consensus Conference – May 2012 recommendations toward standardization

• standardized reference reagents should be developed
  – repository of well-characterized HLA polyclonal and monoclonal reference reagents
    • to all HLA class I and II antigens
    • different titers and isotypes
    • validated in national and international exchange
    • validated on cell panels and solid phase immunoassays
    • validated for all available techniques
  – ongoing technique and reagent validation
  – monitoring interlaboratory variability
  – reproducible quantification of fluorescence values

Tait, et al. Transplantation 2013;95:19
thank you