Navigating Immunoblots
Going Blotto with Blots!

David Gillis
Pathology Queensland
Why am I talking on immunoblots?

• Used extensively in Australia for determination of final auto antibody testing
• Of appeal many autoantibodies measured same time – multiplexing
• Unique auto antibodies measured by immunoblots
• BUT conceptually, anecdotally, evidence wise - limitations to this assay
• Impacting on quality of autoantibody testing
Line Immuno Assays – the myth

- Gold Standard Tests / confirmatory tests
- Regarded as specific and sensitive for disease
- “Used to analyse difficult autoantibody sera”
But the reality!

• Limitations - specificity and sensitivity
• There is no real evidence base for their superior performance
• They are not a gold standard
• They are used inappropriately
• Interpretation is complex - used sequentially with other tests
What do I mean by Immunoblots?

Line Immunoassays
Auto antigens to nylon / solid phase
Auto antigens at high density in line
Serum applied
Second layer
Multiplex Assays
What are immunoblots?

Antigen on separate carriers + Test antibody → Bound antibodies + Enzyme-labeled antihuman Ig antibody → Scanning of substrate conversion

Control

Damioux 2017
Uses of line immunoassays

- ENA antibody - secondary test following ELISA
- Scleroderma blot
- Liver blot
- Myositis blot
- Neuronal blot
- Vasculitis
- Gangliosides (lipid)
Conceptually
Aim of autoantibody assays?

Autoantibody assays should detect antibodies produced by B cells in autoimmune disease.

Surrogate marker for auto reactive B and T cells present in autoimmune disease.

Autoantibody assays detect characteristics pertaining to this set of antibodies.

A larger set of antibodies binds to autoantigens not take part in autoimmune disease.
Characteristics of Pathogenic Autoantibodies

Binding to autoantigen

Bind to autoantigen with high affinity / avidity
Bind to confirmational determinants
Cross linking / precipitation
Bind complement
Functional effects
Characteristics of Autoantibodies measured by traditional techniques

- Indirect immunofluorescence of tissue – binding and confirmational determinants
- Counter immunoelectrophoresis - binding and precipitation and high avidity
- Immuno precipitation - binding, confirmation determinants, precipitation and high avidity
Nature of Autoantibodies detected by Immunoblots

• The nylon column or other solid matrix is to ensure high binding of autoantigen

• Detect all antibodies which bind regardless of other characteristics (pathogenic/non patho)

• Problems with poor specificity and high number of equivocal results
Do altering the cut-offs compensate for the poor specificity

- Compare with line from negative control
- Only accept 2 plus or 3 plus results (calibrate)

- Not always !!!!!! Does not compensate for characteristics of antibody
- Non pathogenic antibodies which just bind can be in high concentration sometimes!
IMMUNOBLOTS – THE MULTIPLEX PROBLEM!

• Disparate auto antibodies measured
• Different conditions for each auto antibody
• Different cut offs for different auto antigens
Further source of variation
Immunoblots

- The actual antigen on the blot, e.g., recombinant, which proteins, purified, linear epitopes
- The way the antigen fixed to the nylon
- The interference and conditions of the processing, e.g., buffer, matrix effects
- Washing and reading – not a robust test!!
- Different lot numbers!!
- Age of sample
Anecdotes!!!
Case 1 - Myositis blot helpful

- 35 year old paint specialist
- Presented with deep ulceration lesions of hands and fingers
- Developed erosive arthritis - PIP and DIP joints
- Noticed to have slightly high CK
- Myositis blot – MDA-5 antibody 3+
Case 2 – More Difficult ? helpful

• 18 years old patient
• Three liver transplants for ductal atresia
• Noticed to have CK 6,000
• Muscle biopsy 2014 normal
• Myositis blot  NXP2 antibody  3 +
• Asymptomatic , no rashes  !!
Case 3 Not helpful

• 72 year old man
• Chronic obstructive airway disease, Bronchiectasis, Reflux - aspiration pneumonia
• Found to have minimal interstitial lung disease
• CK 400 on one occasion
• Myositis Blot Jo-1 ab 2+/ SAE ab 3+
• CI EP- no evidence of Jo-1 antibody

• AND !!
What is the difference between cases?

- The selection of case to be tested based on clinical assessment and other tests
- The results of other test for autoantibodies
- The auto antibody involved
Did the immunoblot really increase diagnostic certainty?

• Or just made us feel better that we were on the right track
• Educate us about a clinical scenario we should have known about anyway
Evidence Base for Problems with Performance of ImmunobLOTS – Specificity

- Positive immunobLOTS in normal patients
- Positive antibodies in well defined patient groups where antibody is not normally found
- “Expansion” of disease associations when measuring antibodies using this method
- Variation between blot manufacturers
Certain autoantibodies perform poorly in line immunoassay

• Ghirardello (2010) - myositis - Ro52 specificity 76% (disease controls)
• Perez (2014) - Ro52 / Ro 60 abs large no of ambiguous results, variability LIA /other tests
• Velsteke et al (2017) – rare myositis antibodies SAE/ TIF-1- gamma antibodies
• Combination- PM/SCI-75 and 100 // RNA pol3 - RP11/155
Immunoblot – Variation

• False negative Th/To antibody as determined by immunoprecipitation

• Some autoantibodies show good correlation with other techniques (fibrillan / Immunoprecipitation / Peterson 2016)
Line blots pick up different patient groups from traditional assays

Mejia et  Respiratory Medicine  123 (2017) 79-86
Bohan and Peter versus IPAF (ILD plus Blot pos)

<table>
<thead>
<tr>
<th>Variable</th>
<th>BPC</th>
<th>IPAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK level</td>
<td>840</td>
<td>87</td>
</tr>
<tr>
<td>Muscle Weakness</td>
<td>97%</td>
<td>43%</td>
</tr>
<tr>
<td>Mechanics Hands</td>
<td>64%</td>
<td>45%</td>
</tr>
<tr>
<td>Fever</td>
<td>85%</td>
<td>58%</td>
</tr>
<tr>
<td>Raynauds</td>
<td>38%</td>
<td>36%</td>
</tr>
<tr>
<td>Sclerodactyly</td>
<td>21%</td>
<td>25%</td>
</tr>
<tr>
<td>DM Rash</td>
<td>21%</td>
<td>3%</td>
</tr>
<tr>
<td>Organising Pneumonia</td>
<td>65%</td>
<td>50%</td>
</tr>
<tr>
<td>NSIP</td>
<td>35%</td>
<td>47%</td>
</tr>
</tbody>
</table>
Line immunoassay Variations

- SM proteins
- RNA polymerase3 antibodies
- Specificity decreases with the increasing number of antigens on the line immunoassay
- Validation both manufacturers and labs on the more prevalent antibodies
Different blots give different results

Yi et al, Clinical Biochemistry 55 (2018) 75-79

<table>
<thead>
<tr>
<th>Cohort</th>
<th>LIA-ANA-Profile -17S</th>
<th>Euroimmune</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE- DsDNA (60)</td>
<td>26%</td>
<td>13%</td>
</tr>
<tr>
<td>SLE- nucleosome</td>
<td>2%</td>
<td>18%</td>
</tr>
<tr>
<td>SLE – Histone</td>
<td>2%</td>
<td>21%</td>
</tr>
<tr>
<td>Ro 52</td>
<td>68%</td>
<td>81%</td>
</tr>
</tbody>
</table>
Summary

• Old pathologists are allowed to present dataless talks at pathology update meetings!!
• Line immunoassays are convenient, multiplex immunoassays BUT
• They have not “solved” the problems of any multiplex assay in autoantibody measurement
Summary

• In particular line immuno assays have
• 1) specificity / (sensitivity) problems
• 2) multiplex problems – one does not fit all
• 3) inter lot variability
• 4) no robust approach to cut off determination
• 5) not robust to subtle differences in technique
• 6) variation from other auto antibody measurement
What to do?

- External QAP = target should not depend on technique used by greatest number of users
- Line immunoassay interpreted with other techniques eg indirect immunofluorescence
- Determination of specificity of every autoantibody (line) measured
- Not used as a gold standard for difficult cases but as additional technique with limitations
- Controls should be expanded
Fig. 1 IIF on HEp-2 ANA slides of myositis-specific autoantibodies from patients with IIMs (EUROIMMUN, Lübeck, Germany). a Anti-Jo-1 and b anti-Jo-1 and anti-Ro52, c anti-PL-7, d anti-PL-12, e anti-KS, f anti-Mi-2, g anti-SAE-1, h anti-MDA5, i anti-NXP-2 and j anti-NXP-2 with coiled bodies, k anti-TIF1γ, l anti-SRP