Overview of assays for influenza vaccines immunology evaluation and correlates of protection
Unresolved Issues in Influenza

• Understand pathogenesis and transmission of avian influenza to mammals
• Understand immunologic correlates of protection from disease
• Improve the effectiveness of influenza vaccines
• Improve vaccine production
• Eliminate disparities in vaccination
• Determine risks and benefits of universal vaccination
• Determine and implement cost-effective methods for adverse vaccine event reporting
What is a correlates of protection?

Correlates of protection
• An immune response that is responsible for and statistically interrelated with protection

Surrogate correlate of protection
• An immune response that substitutes for the true immunological correlates of protection, which may be known or not easily measured
Antibody concentration in serum, units per ml

Time after immunization

1° Ag  Naïve lymphocytes
2° Ag  Memory lymphocytes

Primary response
Secondary response

Total
IgG
IgM

Higher magnitude response
Higher affinity (IgG)

Large population memory cells, easily activated
Questions to be asked of the experts

- What evidence-based criteria for regulatory evaluation of candidate pandemic influenza vaccines can be implemented immediately. What research would be necessary to improve assessment of efficacy (immunogenicity) of influenza pandemic vaccines within the next 2-3 years?

- What assays, and/or experimental systems, should be used to evaluate candidate pandemic influenza vaccines?
2.5 Antibody titration
All sera shall be assayed for anti-hemagglutinin antibody against the prototype strains by HI (Palmer et al., 1975) or SRH (Schild et al., 1975, Aymard et al., 1980) tests.

Positive and negative sera as well as reference preparations may be obtained from a reference laboratory.

2.6 Interpretation of results and statistics
Antibody titrations shall be done in duplicate; pre- and post-vaccination sera shall be titrated simultaneously.

The titre assigned to each sample shall be the geometric mean of two independent determinations.
Serological Assay for Abs detection - Characteristics

HAI – Haemagglutination Inhibition
- Suitable for screening a large number of samples
- Good correlation with MN for seasonal strains
- BSL2 lab need
- EMA and FDA Approved

SRH – Single Radial Haemolysis
- Suitable for screening a large number of samples
- Good correlation with MN for pandemic strains
- BSL2 lab need
- EMA Approved

MN – Virus Neutralization
- Titration of functional antibody only
- Gold Standard for confirmation
- Not easy for screening a large number of samples
- High containment (BSL3plus) needed in case of pandemic strains
- Automation is possible

ELISA – Enzyme Linked ImmunoSorbent Assay
- Suitable for screening a much larger number of samples
- Automation is possible
- No correlate of protection
- Use of HA is preferable ..... HA1 is better

WB – Western Blot
- Useful only for confirmation
Haemagglutination Inhibition (HAI)

- Heat to inactivate complement in serum
- Add Receptor
  Destroy Enzyme
- Adsorb to RBC, then remove
- Dilute (1/20, 1/40 etc)
- Add virus
- Incubate
- Add RBCs
- Incubate
HI assay
(haemagglutination inhibition assay)

- Influenza HA binds to sialic acid receptors on surface of erythrocytes
- HI assay measures the ability of serum antibodies to inhibit haemagglutination

<table>
<thead>
<tr>
<th>No agglutination</th>
<th>50% Agglutination</th>
<th>100% Agglutination</th>
</tr>
</thead>
</table>

- Surrogate correlate of protection: HI titre $\geq$ 40 for seasonal vaccines
Hemagglutination inhibition (HAI)

Hemagglutination inhibition (HAI) and MN, correlation for seasonal strains.

Relationship between HI and neutralizing-antibody (N) titers against A/Yamagata/120/86 (H1N1) (A), A/Fukuoka/C29/85 (H3N2) (B), and A/Shisen/2/87 (H3N2) (C).

Okuno, J Clin Microbiol. 1990
Haemagglutination inhibition (HAI), correlation for seasonal and pandemic strains.

Comparison Neut / HI H3N2 vaccine sera

Comparison Neut / HI H5N3 vaccine sera

From: M. Zambon, HPA London
Haemagglutination inhibition (HAI), H5 SENSITIVITY

The hemagglutination inhibition (HI) titer of 1:40, which has been recognized as an immunologic correlate corresponding to a 50% reduction in the risk of contracting influenza, is based on studies in adults. Neither seasonal nor challenge-based correlates have been evaluated in children.

A recent meta-analysis of the relationship of HI and clinical protection in adults supported the use of a 1:40 titer as a correlate of 50% protection in adults, although the supporting data from clinical trials in the meta-analysis were weak. However, data in this paper do not support the use of a 1:40 titer as a correlate of 50% protection against influenza infection in children less than 6 years of age. A cut-off of 1:110 measured 21 days after the second vaccine dose may be used to predict a 50% clinical protection rate in this age group.

The reason that more antibody was required for protection in children is not known, several factors might contribute to the need for more antibody to provide protection in children. The most important is probably the fact that the younger the age of the individuals the lower the probability that a child has had immunologic experience (induction of cell-mediated immunity, specific memory) with influenza either through vaccination or infection.

<table>
<thead>
<tr>
<th>Probability of Protection</th>
<th>H3N2 Antibody Titer Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>1:110</td>
</tr>
<tr>
<td>60%</td>
<td>1:151</td>
</tr>
<tr>
<td>70%</td>
<td>1:215</td>
</tr>
<tr>
<td>80%</td>
<td>1:330</td>
</tr>
<tr>
<td>90%</td>
<td>1:629</td>
</tr>
</tbody>
</table>

Black S. PIDJ 2011
Single radial haemolysis (SRH) is routinely used for the detection of influenza-specific (and rubella) IgG antibody.

- SRH has been shown to be sensitive, specific, and reliable.
- SRH plates are usually prepared in the laboratory using commercially available reagents.
- Test sera are placed in wells on a plate containing agar with influenza antigen-coated RBC and guinea-pig complement.
- The presence of influenza-specific IgG is detected by the lysis of influenza antigen-coated RBC mediated from GUINEA-PIG complement.
- The zone of lysis around the well is dependent on the level of specific antibody present.


• The size of the haemolysis zone around a well containing serum is measured in mm. The diameter of haemolysis is then transformed in area.
• If the area size is greater than 25 mm\(^2\), then the subject is considered to be seroprotected in accordance with EMEA guidelines.
• If the area size is \(\leq 4\) mm\(^2\), then the subject is considered negative according to EMEA guidelines.
SRH - procedure:

...... we measure the diameters of haemolisys around each hole in millimetres. This procedure should be done manually whit the support of millimetres paper (fig. 1) or through an instrument (fig. 2) called “Calibrating Viewer Transidyne” that give the measure of haemolisys diameters in millimetres
SRH – for pandemic vaccine

Will the SRH method be adequate for the study of pandemic vaccines? A/H5N1, A/H5N3, A/H7N1, .......

A single radial haemolysis assay for antibody to H5 haemagglutinin

J.M. Wood\textsuperscript{a,*}, D. Melzack\textsuperscript{a}, R.W. Newman\textsuperscript{a}, D.L. Major\textsuperscript{a}, M. Zambon\textsuperscript{b}, K.G. Nicholson\textsuperscript{c}, A. Podda\textsuperscript{d}

- It has been recognised that the haemagglutination–inhibition (HI) test is not sufficiently sensitive to detect human antibody to A/H5N1 influenza virus.
- A modified single radial haemolysis (SRH) test has been described, the SRH immunoplates were prepared as described by Schild using turkey erythrocytes.
- The SRH test recognised the antibody in a specific antiserum to A/H5N1/Hong Kong/489/97 virus, however rabbit antibody to an A/H10N7 avian virus and human antibody to A/H1N1 or A/H3N2 viruses did not react.
- H5 SRH antibody induced by human A/H5N1 virus infection and A/H5N3/Duck/Singapore/97 vaccination correlated with antibody detected by MN techniques.
- The modified SRH test offers a good serological technique for detecting antibody to H5 haemagglutinins.
A single radial haemolysis assay for antibody to H5 haemagglutinin


This study has also demonstrated that caution must be exercised in planning and interpreting SRH tests. The H5 SRH test could detect non-specific antibody in human sera, but the antibody could be removed by adsorption with other influenza A viruses. It is likely that the cross-reactivity is due to antibody induced by influenza A internal proteins. Therefore, for SRH detection of antibody to influenza A viruses, a preliminary screen for cross-reactivity, a confirmatory test with an alternative serological technique or a virus adsorption step are recommended.

One thousand three hundred and sixty-four samples of sera gathered from subjects in Italy from 1992 to 2007 were studied. Of patients in the range of age between 18 and 64 years, 5.98 % were seroprotected against the A/H5N1/Vietnam/1194/2004 virus, and this was also true for 12.99 % of subjects of over 65 years of age. The seroprotected subjects were analysed again in order to remove non-specific antibodies, and these samples were adsorbed in a 1:1 vol mixture of A/H1N1/New Caledonia/20/99 and A/H3N2/California/7/2004 viruses (2000 UE/ml). Of these, 53.57 % of the patients passed from seroprotected to negative after this test.
Percentage of influenza A/H5N1/Vietnam/1194/2004 seropositivity based on year collected samples.

The percentage of seropositivity for each age group is indicated.

The number of serum samples positive / tested in each age group is indicated.
SRH / H5 – sensitivity

Galli et al. PNAS 2009
Laboratory Y is Reference Lab for SRH

A/H1N1/Solomon Islands/3/2006

A/H3N2/Wisconsin/67/2005

B/Malaysia/2506/2004

BSP063 - Influenza - Collaborative Study - Biological Standardisation Programme - Council of Europe, EDQM
CDC Neutralisation Test

1. Treat sera
   56°C 30 min/RDE

2. Add Sera

3. Dilute sera

4. Add Virus
   100 TCID\textsubscript{50}/well

5. MDCK Cells
   1.5x10\textsuperscript{4} cells/well

6. Fix cells and run NP ELISA
   16-22 hr @37°C

From: Rowe T. J Clin Microb 1999
Neutralisation Test

Evaluation of Results

• by CPE for each well:

• by HI in supernatant of each well:

• by ELISA-NP in each well:

Collaborative studies to evaluate reproducibility of flu serology assays

Independent study

• 1994 – Seasonal H1N1, H3N2, B viruses; HI and SRH (Wood et al, 1994)

WHO studies

• 2007 – H3N2 virus; HI and VN (Stephenson et al, 2007)
• 2009 – H5N1 virus; HI and VN (Stephenson et al, 2009)
• 2009-10 – H1N1pdm; HI and VN (Wood et al, manuscript in preparation)

EU studies

• 2007-9 – Seasonal H1N1, H3N2, B viruses; HI and SRH (EDQM BSP063)
• 2009-10 – Retesting of sera from pandemic influenza vaccine trials; HI and VN (Wagner et al, manuscript in preparation)
Between laboratory reproducibility was poor

HI assay
- Median GCV 112-261% (16-128-fold)

SRH assay
- Median GCV 57% (3.8-fold)

VN assay
- Median GCV 175-359% (35-724-fold)

EDQM study – variability affected compliance with licensing criteria
MN for Pandemic Assay using PP

3 plasmid transient transfection into 293T cells

Haemagglutinin (HA)
Lentiviral Gag-Pol
Vector encoding luciferase

Measure N-Ab titre of serum using a luciferase readout

Vaccine 27 (2009) 5998–6003

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Pseudoparticle neutralization is a reliable assay to measure immunity and cross-reactivity to H5N1 influenza viruses

Isabella Alberini a, Elena Del Tordello a, Alba Fasolo a, Nigel J. Temperton b, Graziella Galli a, Chiara Gentile c, Emanuele Montomoli c, Anne K. Hilbert d, Angelika Banzhoff d, Giuseppe Del Giudice a, John J. Donnelly a, Rino Rappuoli a, Barbara Capechi a
Comparison of MN PP-based with HI, SRH and MN titers. Scatterplots showing the correlation of antibody logarithmic titers measured by PPN versus HI (A), SRH (B) and MN (C) assays performed against the vaccine strain Vietnam/1194/2004.

Alberini et al. Vaccine 2009
Less cross-reactivity when only HA1 and not HA2 is used in ELISA?
### ELISA Results

#### HA

<table>
<thead>
<tr>
<th>HA</th>
<th>VN</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td>ELISA</td>
<td>Neg</td>
<td>161</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Pos</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>190</td>
<td>27</td>
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</table>

**Sens. = 0.70**  
**Spec. = 0.85**

#### HA1

<table>
<thead>
<tr>
<th>HA1</th>
<th>VN</th>
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</thead>
<tbody>
<tr>
<td>ELISA</td>
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<td>Pos</td>
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<tr>
<td>Total</td>
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<td>190</td>
<td>27</td>
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**Sens. = 0.70**  
**Spec. = 0.96**

#### HA

<table>
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<tr>
<th>HA</th>
<th>SRH</th>
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<tbody>
<tr>
<td>ELISA</td>
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<td></td>
<td>Pos</td>
<td>28</td>
<td>20</td>
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<tr>
<td>Total</td>
<td></td>
<td>177</td>
<td>40</td>
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</table>

**Sens. = 0.50**  
**Spec. = 0.84**

#### HA1

<table>
<thead>
<tr>
<th>HA1</th>
<th>SRH</th>
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<tbody>
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<td>ELISA</td>
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<td></td>
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<td>6</td>
<td>20</td>
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<tr>
<td>Total</td>
<td></td>
<td>190</td>
<td>27</td>
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**Sens. = 0.50**  
**Spec. = 0.97**

#### HA

<table>
<thead>
<tr>
<th>HA</th>
<th>HI</th>
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<tbody>
<tr>
<td>ELISA</td>
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<td>37</td>
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<tr>
<td>Total</td>
<td></td>
<td>204</td>
<td>13</td>
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</table>

**Sens. = 0.81**  
**Spec. = 0.85**

#### HA1

<table>
<thead>
<tr>
<th>HA1</th>
<th>HI</th>
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<tbody>
<tr>
<td>ELISA</td>
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<tr>
<td></td>
<td>Pos</td>
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<td>12</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>204</td>
<td>13</td>
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</tbody>
</table>

**Sens. = 0.93**  
**Spec. = 0.92**

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From: M. Zambon, HPA London
NA is important in release of virus

It is in inactivated vaccines

Neuraminidase-inhibiting antibodies can reduce viral spread and may be of particular importance in the event of an H5N1 pandemic, where immunity due to HA antibodies is likely absent in the population.

2 ASSAYS:

- NA inhibition assays based on thiobarbituric acid (TBA-based assay)
- Enzyme-linked lectin NA inhibition assay (ELLA)

\[ r = 0.83 \]
\[ P < .0001 \]
## NA Important in release of virus

Table 1. Proportion of Subjects With Seroconversion and Geometric Mean of the Increase From Baseline Based on Antibodies Against H5 and N1

<table>
<thead>
<tr>
<th>Dose</th>
<th>Assay</th>
<th>Seroconversiona</th>
<th></th>
<th>GMI Value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 21</td>
<td>Day 42</td>
<td>Day 21</td>
<td>Day 42</td>
</tr>
<tr>
<td>7.5 µg</td>
<td>TBA</td>
<td>23.8 (12.1–39.5)</td>
<td>26.6 (15.7–44.6)</td>
<td>2.1 (1.7–2.7)</td>
<td>2.1 (1.6–2.9)</td>
</tr>
<tr>
<td></td>
<td>ELLA</td>
<td>69.9 (43.3–74.4)</td>
<td>64.3 (48.0–78.4)</td>
<td>5.3 (3.8–7.4)</td>
<td>5.3 (3.7–7.6)</td>
</tr>
<tr>
<td></td>
<td>MNp</td>
<td>35.7 (21.6–52.0)</td>
<td>69.0 (52.9–82.4)</td>
<td>5.3 (4.1–6.9)</td>
<td>5.3 (4.1–6.9)</td>
</tr>
<tr>
<td></td>
<td>SRHp</td>
<td>61.9 (45.6–76.4)</td>
<td>73.8 (58.0–86.1)</td>
<td>4.8 (3.2–7.2)</td>
<td>6.3 (4.3–9.1)</td>
</tr>
<tr>
<td>15 µg</td>
<td>TBA</td>
<td>19.5 (8.8–34.9)</td>
<td>30.0 (24.2–55.5)</td>
<td>2.1 (1.6–2.6)</td>
<td>2.8 (2.2–3.6)</td>
</tr>
<tr>
<td></td>
<td>ELLA</td>
<td>53.7 (37.4–69.3)</td>
<td>63.4 (46.9–77.9)</td>
<td>4.6 (3.5–6.0)</td>
<td>5.2 (4.0–7.0)</td>
</tr>
<tr>
<td></td>
<td>MNp</td>
<td>34.9 (21.0–50.9)</td>
<td>68.3 (51.9–81.9)</td>
<td>3.1 (2.5–4.0)</td>
<td>5.7 (4.3–7.5)</td>
</tr>
<tr>
<td></td>
<td>SRHp</td>
<td>39.5 (25.0–55.6)</td>
<td>58.5 (42.1–73.3)</td>
<td>2.8 (1.9–4.2)</td>
<td>4.7 (3.1–7.1)</td>
</tr>
</tbody>
</table>

Fritz, JID 2011
Challenges for correlates of protection

- Need for HI titres to be associated with laboratory confirmed influenza (culture and PCR)
- Need for definition of HI / SRH and MN titres associated with protection for pandemic strains
- Need for well defined correlates of protection (H5, H7 and pediatric population)
- Validated, standardized assays to reduce laboratory variation (Protocols, reagents and international antibody standard)
- Head to head comparison of vaccines
- Development of novel standardized assays for novel vaccines
Acknowledgment

- Ilaria Manini
- Giulia Lapini
- Simona Piccirella

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