Correlates of Protection for Flu vaccines and Assays Overview

by Simona Piccirella, PhD
Chief Executive Officer
VisMederi is an Italian private small enterprise established in 2009 and engaged in Life Sciences and especially in the Serology field, that usually provides microbiological analytical services to the Pharmaceutical Industry.

VisMederi’s expertise focuses on early and later phases of method development, method validation and serological assays associated with Immunogenicity and Cell Mediated Immunity.

Experience in Serology Assays:

VisMederi provided serology assays (SRH, HI, MN tests) for several clinical trials overall for Flu vaccine licencing (Plant-Made H1 and H5; Subunit; Vero-Derived whole virus; MDCK-derived); Ab detection for Measles, Polio virus (type 1, type 2, type 3), Tethanus, Rubella (by ELISA and Neutralization assay) and Meningococcal Antigen Typing System (MATS) for Neisseria meningitidis unknown strains.
What is a correlate of protection?

**Correlate of protection**
- An immune response that is responsible for and statistically interrelated with protection
- All individuals protected or population average e.g. 50%?

**Surrogate correlate of protection**
- An immune response that substitutes for the true immunological correlates of protection, which may be known or not easily measured

i.e. non mechanistic correlate of protection, which does not cause protection but nevertheless predicts protection through its (partial) correlation with another immune response that mechanistically protects).

**Differ-vaccine type and formulation, age, health status**

*by Plotkin S, Clinical and Vaccine Immunology, 2010*
Correlate of protection

«At present, widely accepted immunological correlates of protection exist for certain antigens only and consist of defined humoral antibody responses, above which there is a high likelihood of protection in the absence of any host factors that might increase susceptibility to the infectious agent.»

Guideline on clinical evaluation of new vaccines (EMA/CHMP/VWP/164653/2005)
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.

b) in HI tests, seroconversion corresponds to:
   - negative prevaccination serum / postvaccination serum ≥ 40;
   - a significant increase in antibody titre, i.e. at least a fourfold increase in titre;

c) in SRH tests, seroconversion corresponds to: (*)
   - negative prevaccination serum / postvaccination serum: area ≥ 25 mm²;
   - a significant increase in antibody titre, i.e. at least a 50% increase in area;
### CHMP Criteria (EU)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>18-60 years old</th>
<th>60 years old</th>
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<tbody>
<tr>
<td>Seroprotection Rate (SRP)</td>
<td>&gt;70%</td>
<td>&gt;60%</td>
</tr>
<tr>
<td>(HI titre ≥ 40 or SRH &gt; 25mm²)</td>
<td></td>
<td></td>
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<tr>
<td>Seroconversion Rate (SCR)</td>
<td>&gt;40%</td>
<td>&gt;30%</td>
</tr>
<tr>
<td>Mean Geometric Titre Increase (GMT)</td>
<td>&gt;2.5</td>
<td>&gt;2</td>
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**EMA (CHMP):**
- **Seasonal vaccines:** must meet at least one of the three parameters;
- **Pandemic vaccines:** all three parameters must be met.

### CBER Criteria (ES)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subjects less than 65 years</th>
<th>Adults ≥ 65 years</th>
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<tbody>
<tr>
<td>Lower limit of two-sided 95% CI for Seroprotection</td>
<td>&gt;70%</td>
<td>&gt;60%</td>
</tr>
<tr>
<td>Lower limit of two-sided 95% CI for Seroconversion</td>
<td>&gt;40%</td>
<td>&gt;30%</td>
</tr>
</tbody>
</table>

**FDA:**
- **Seasonal vaccines:** lower bound of 95% CI ≥ criterion (GMT and seroconversion)
- **Pandemic vaccines:** lower bound of 95% CI ≥ criterion for all parameters
HI assay
(Hemagglutination Inhibition Assay)

- Suitable for screening a large number of samples
- Detects Ab that bind around receptor-binding site globular head and block agglutination
- EMA and FDA Approved

HI assay detects HA-Ab by hemagglutination of RBCs;
Influenza HA binds to sialic acid receptors on surface of erythrocytes: hemaglutination
HI assay measures the ability of serum antibodies to inhibit hemagglutination
Correlate of protection: HI titre ≥ 40 for seasonal vaccines
HI assay  
(Hemagglutination Inhibition Assay)
HI assay
(Hemagglutination Inhibition Assay)

Single Radial Hemolysis (SRH) assay

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 is EMA approved.
- SRH plates are usually prepared 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 hemolysis area around the well depends on the level of specific antibody present.


Single Radial Hemolysis (SRH) assay

- 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 ≥ 25 mm², then the subject is considered to be seroprotected in accordance with EMA guidelines.
- If the area size is ≤ 4 mm², then the subject is considered negative according to EMA guidelines.
we measure the diameters of hemolisys around each hole in millimetres. This procedure should be done manually by an instrument (fig. 1-2) called “Calibrating Viewer Transidyne” that gives the measure of hemolisys diameters in millimetres or using graph paper (fig. 3).
SRH assay– for Flu 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

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\begin{itemize}
\item It has been recognised that the haemagglutination–inhibition (HAI) test is not sufficiently sensitive to detect human antibody to A/H5N1 influenza virus.
\item A modified single radial haemolysis (SRH) test has been described, the SRH immunoplates were prepared as described by Schild using turkey erythrocytes.
\item 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.
\item H5 SRH antibody induced by human A/H5N1 virus infection and A/H5N3/Duck/Singapore/97 vaccination correlated with antibody detected by MN techniques.
\item The modified SRH test offers a good serological technique for detecting antibody to H5 hemagglutinins.
\end{itemize}
SRH – for Flu pandemic vaccine

A single radial haemolysis assay for antibody to H5 haemagglutinin

J.M. Wooda,*, D. Melzacka, R.W. Newmana, D.L. Majora,
M. Zambonb, K.G. Nicholsonc, A. Poddad

This study has also demonstrated that caution must be exercised in interpreting SRH tests.

The SRH test for H5 Flu virus 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 assay or a virus adsorption step are recommended.
1364 serum samples gathered from subjects in Italy from 1992 to 2007 were studied.

5.98% of patients aged between 18 and 64 years old were seroprotected against the A/H5N1/Vietnam/1194/2004 virus and this was also true for 12.99% of subjects aged over 65 years old. The seroprotected samples underwent a second SRH analysis in order to remove the 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).

53.57 % of those samples passed from seroprotected to negative after this test.
Immunological assessment and criteria

Clinical studies should provide a detailed characterisation of immunological responses to the strain in the candidate influenza vaccine, which should be the strain intended for the final product. Data generated during clinical studies conducted with vaccines manufactured similarly but containing other influenza viruses, including other strains with a potential to cause a pandemic or seasonal influenza strains, may be considered to be supportive.

The comprehensive results from the HI, SRH and microneutralisation assays will form the basis for the assessment of immunogenicity. The choice of methodology and the standardisation of the assays should be addressed by the applicant. Applicants should predefine in the protocol which immunological parameter(s) will be used in the primary analysis of immunogenicity.

However, with no other criteria to suggest at present, it is anticipated that mock-up vaccines should at least be able to elicit sufficient immunological responses to meet all three of the current standards set for existing vaccines in adults and older adults >60 years (CPMP/BWP/214/96) based on haemagglutination inhibition (HI) and/or serum radial haemolysis (SRH).

If these criteria are not met the applicants are urged to further support the immune responsiveness of the vaccine using other assays, such as neutralising antibody assays and if possible, explore cell mediated immunity.

It is expected that serum neutralising antibody (SNA) will also be assessed, at least in a subset of vaccines. In addition applicants are encouraged to measure anti-neuraminidase antibody and to investigate the cell mediated immunity elicited by the mock-up strain vaccine, although these responses are still of unknown relevance to protection. However exploratory data in a subset of vaccines after primary and booster vaccination may provide additional insight into the overall effects of vaccination and the potential usefulness of the vaccine (early) in a pandemic.

For HI, SRH and SNA it is recognised that there is considerable intra- and inter-laboratory variation in methodology and that the actual titres that might be reported from a range of samples can be very different. EU regulators are aware that there are efforts ongoing to improve on assay variability and international standards are likely to be developed. It is expected that applicants provide full details of assay validation and controls and that the assays are updated and improved in the light of any new developments and availability of international standards. The applicant is recommended to keep reference serum for future analyses and standardisation.
NEUTRALIZATION TEST

1. Complement heat inactivation of the serum samples for 30 minutes at 56°C.

2. Add and dilute the sera.

3. Add virus

4. 1 hour @ 37°C

5. Add MDCK cells (1.5 x 10^5 cells/ml).

6a. 5 days @ 37°C

6b. 16-22h @ 37°C

7a. Read out by cpe evaluation

7b. Read out by NP-ELISA

- Detects Ab that bind around globular head and block virus attachment/entry
- May detect cross-reactive antibody including HA stem
- Not easy for screening a large number of samples
- High containment (BSL3plus) needed in case of pandemic strains
- No correlate of protection established
Hemagglutinin inhibition assay
Detects Ab that bind around receptor-binding site globular head and block agglutination

Single Radial Haemolysis assay
Detect Ab-HA involved in complement mediated reaction

Microneutralization test
Detects Ab that bind around globular head and block virus attachment/entry

Is there a correspondance between the outcomes obtained by these different assays?
HI assay and Microneutralization test: correlation for FLU seasonal strains.

Relationship between HAI 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
HI assay: sensitivity toward H5 Flu pandemic strain

Stephenson et al., 2004
SRH / H5 – sensitivity

Galli et al. PNAS 2009
The hemagglutination inhibition (HAI) 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 HAI 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.
Lentiviral Gag-Pol

Haemagglutin (HA)

Vector encoding luciferase

Measure N-Ab titre of serum using a luciferase readout

3 plasmid transient transfection into 293T cells

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, Grazia 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 Capecchi a
Comparison of MN PP-based with HAI, SRH and MN titers. Scatterplots show the correlation of antibody logarithmic titers measured by PPN versus HAI (A), SRH (B) and MN(C) assays performed against the vaccine strain A/H5N1/Vietnam/1194/2004.

Alberini et al. 2009
Neuraminidase-inhibiting antibodies can reduce viral spread and may be of particular importance in the event of an H5N1 pandemic, where immunity due to H5 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)
The highly significant correlation of NA antibody titers determined by the ELLA and HA-specific neutralizing antibody titers as determined by MN assays of the same serum samples, described in the current study, suggests that the NA and HA components of the H5N1 vaccine were similarly immunogenic in the study population. This finding is further supported by the comparable GMI values calculated from results of the ELLA and the MN assay.
Challenges for correlates of protection

- Need for definition of HI / SRH and MN titres associated with protection for Flu pandemic strains
- Need for HI titres to be associated with laboratory confirmed influenza (culture and PCR)
- Validated, standardized assays to reduce laboratory variation (Protocols, reagents and international antibody standard)
- Development of novel standardized assays for novel vaccines
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Professor of Public Health and Responsible of Epidemiology Molecular Lab (Univ. of Siena)