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Report from scientific workshop on serology assays and correlates of protection for influenza vaccines

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Table of content

1. Executive summary, list of actions to be considered

From the various meetings and teleconferences which have been held in the past twelve months to consider the "serology" question for influenza vaccine, the following actions should be considered, in a stepwise approach. A tentative priority list of actions is proposed below, which should be further elaborated with the concerned stakeholders as some proposals are for the research/discovery field whereas others can be part of maintenance and improvement of the marketing authorisation dossiers.

1.1. Serological tests

The conventional HI, VN, SRH serology assays are the only assays currently available for routine and wide scale assessment of influenza vaccine immunogenicity and should currently be retained in the battery of tests for acceptance criteria for flu vaccines.

1.1.1 HI and SRH: most widely used assays to quantify antibody to HA (see correlate section). Several collaborative studies indicate that those assays are reproducible within but variable between laboratories.

Further assay standardisation is needed to reduce the variability. The following actions should be encouraged:

a) to use standardised procedures, protocols and reagents (particularly source of erythrocytes depending on the strain tested);

b) to monitor technical proficiency

c) to use SRH in preference to HI

- ⇒ for influenza B assays;
- ⇒ for serological assay in paediatric populations as SRH measures only IgG

d) to use the Beyer correction for pre-vaccination antibody titres;

e) to issue guidelines on selection of clinical trial serum panels.

f) Encourage the use of an "antibody standard" (international antibody standards, regional standards or panels of sera) any time a new vaccine composition is tested in humans. The feasibility to produce and use antibody standards for seasonal influenza vaccines needs to be explored.

g) Possibly use central laboratories for retesting subset of samples

1.1.2 This interlab assay variability affects compliance with CHMP criteria so that for any given clinical trial, one laboratory could obtain serology data which comply, whereas another laboratory could obtain data which fail. In addition the assay variability could affect the ability to compare different vaccines and the ability to develop meaningful correlates of immunity. Thus for seasonal vaccines, where only one out of the three CHMP criteria has to be met, it is necessary to reconsider, even before any other criteria, the need or not for clinical trial(s) for the annual update or at least to propose a more informative trial.

1.1.3 The CHMP criteria should be re-established after correlates of protective immunity have been re-evaluated from laboratory-confirmed cases (PCR or virus isolation) of influenza infection. It is acknowledged that this may take several years to achieve so in the interim, the following measures are recommended.

a) The CHMP criteria should include HI, SRH and VN assay data

b) The relevance of the 'protective rate' CHMP criteria needs to be re-evaluated

c) The CHMP criteria should include a statement relating to assay variability and assay standardisation

d) CHMP criteria should include a statement on the relevance of antibody to NA

1.1.4 an important serology assay : Because the HI assay has recognised deficiencies and as its functional activity may not entirely reflect biological relevance to protective immunity, it is desirable to supplement HI assay data by use of more functional assays such as VN. It is important to identify targets for virus neutralisation and the epitopes implicated in protection. Antibody to virus NA is important in preventing replicated viruses to release from cells and the role in protective immunity needs to be better understood by use of suitable VN assays (development of NA pseudotype release assays in conjunction with the newly developed neuraminidase-inhibition (NI) assays). Miniaturised TBA or Enzyme-linked lectin assay (ELLA), could be envisaged as they are also capable of analysing the specificity of antibody binding to NA and thus have the potential to examine NA antigenic drift and measuring the antibody to NA induced by vaccines. Finally, if the NA content of future vaccines becomes an important parameter, assays for the NA content of influenza vaccines should also be developed and validated.

1.1.5 Assays to detect antibody to M2e protein; the conserved regions of HA (eg HA2) and Th1 or Th2 directed immune responses will lead to a better understanding of molecular substrate(s)/target(s) of the protective immunity.

1.1.6 As regards cell-mediated immunity (CMI):

a) It is recognised that CMI assays are still in the exploratory phase and there should be attempts to reach consensus on the most appropriate assays before recommending greater use and initiate assay standardisation

b) The use of validated CMI assays should be encouraged in vaccine studies, with the view of establishing, if proved useful, new immunological criteria as surrogate for vaccine efficacy.

c) Attention should be paid to the development and validation of assays to analyse the CMI, importance of which in protection against influenza is still being elucidated.

1.2. Clinical correlates

As regards clinical correlates of protection the main messages and recommendations are as follows:

1.2.1 Clinical correlates for protection: the first question is to define the objective of "protection" for an influenza vaccine. Do we need to protect against infection (sterilising immunity), (severe) illness and hospitalization or fatal outcome. Regulatory agencies should take proactive initiatives and advise manufacturers to consider measuring, in a prospective approach, other potentially relevant responses in addition to HI/SRH. Anti HA response could be considered as a protection against infection, whereas anti NA response could be considered (in analogy with antiviral agents acting as neuraminidase inhibitors) as a protection against the disease. This is of importance for the future strategy in preparation to a new pandemic phase, which may be caused either by a new NA type that has never been circulating or a NA type that has been circulating as seasonal strains.

1.2.2 The precise determinant(s) of the serological correlates to clinical protection (in the meaning of "no clinical disease") are not well understood and therefore the exact serological post-vaccination parameters to be measured are not known.

a) The HI serological results (which describe the response against HA) is well correlated in adult and elderly, although the precise role of antibodies measured by HI in protection should be revisited using contemporary endpoints (PCR confirmed by cell culture isolation).

b) VN assays offer the most relevant and sensitive measure of post-vaccination antibody but need clinical correlation by field studies before they can be considered by regulatory authorities as additional acceptance criteria

c) Epitope mapping is important to understand the targets for neutralising antibody to influenza, and identifying potential new seroconversion or seroprotection markers

d) Serological correlates are different with the various vaccine formulations and particularly for live attenuated vaccines for which current CHMP criteria do not apply as there is a lack of correlation of serum antibody to HA after vaccination with LAIV

1.2.3 Further research should be encouraged to investigate additional antigens of possible importance for protection, e.g. NP, M1, M2e and on other elements of the immune responses to be considered such as CTL, T helper cells and mucosal response.

1.2.4 For paediatric population, in contrast with the adult/elderly, the picture is not clear at all

a) Is the response to HA (measured by HI or SRH assay) the best parameter to monitor priming and boosting of children?

b) A booster dose needs to be given in all primary vaccination strategy in order to ensure priming to NA

c) Considering the results of the Murphy trial (Murphy, NEJM 1972; 286:1329) it could be recommended for development of new flu vaccines for children, that manufacturers:

⇒ Measure the NA content of the vaccine

⇒ Investigate serological determinants of the anti-HA and anti-NA response. For the NA response the VN assay, using the long-incubation time could be, for the time being and considering the experience gained with this assay, the best option be used in the clinical trials.

d) Adjuvanted TIV is needed in priming but dose-finding studies have to be performed for determining the best adjuvant to antigen ratio.

1.2.5 Other avenues to be investigated in the immune response and attempt to correlate with clinical protection:

a) Specific B and T cells can represent a very good alternative to antibody quantification to identify priming events in immunologically naive individuals. However, despite the impressive technological progress made in the past few years in this field, measurement of B- and T-cell responses still faces several hurdles: standardization of cell preparation, timing of blood draw, reproducibility/inter-laboratory comparability, volumes of blood (an issue in paediatric trials) and costs.

b) Current serological assays do not identify all primed individuals and also that human challenge studies could be used to answer many basic questions in immune response, clinical protection and correlates of protection

2. Introduction

In the vaccine field, the main question has always been, how to measure and ascertain the immune response to the vaccination and conclude on "protection" against any wave of circulating pathogenic agent? This question has become more acute for influenza vaccine for which several problems have been identified in the past years:

- What should be measured (antibodies to HA, NA, IgM, IgG, neutralising activity, Cell mediated immunity)?
- What serological test should be used (depending on what is to be measured)?
- What limits (criteria) should be set for each of those tests applied?
- What criteria for what age population?
- Should the criteria differ for seasonal or pandemic scenario?

In the last 10 years these questions have emerged repeatedly and the serology assay problems have been identified as they were calling into doubt the usefulness and relevance of the yearly clinical trials imposed to verify the "efficacy" of the new composition. Collaborative studies have been initiated, in an attempt to reduce the inter- and intra-lab variability, but still the relevance of the serology results was questioned.

It was in this context of uncertainty that the reflections on the pandemic scenario started, with the preparedness plan and the concept of mock-up vaccines.

Furthermore the 2009-2010 pandemic started, not with the expected H5N1 candidate, on which the mock up strategy had been elaborated and the vaccines developed, but with an A,H1N1 strain, which was claimed not to cross react with the seasonal H1N1 strain.

When the first clinical trial results for H1N1 pandemic strain became available, the CHMP experienced difficulties in their interpretation, due to a large inter-manufacturers variability. Due to this variability, it has been difficult to make valid recommendations in terms of dosage schedule for the respective vaccines and respective populations to be vaccinated.

Therefore, in summer 2009, at an EMA serology meeting it was decided to initiate a "retesting" exercise with two EDQM central labs using haemagglutination inhibition (HI) and virus-neutralisation (VN) assays to retest some samples collected in the clinical trials conducted by the various pandemic vaccine manufacturers.

This was the first step of this initiative for reconsidering the "serology question for influenza vaccine"

As part of the "lessons learnt" exercise, and as a follow up to discussions in 2009 on serology assays, this scientific workshop on "serology assays and correlates for protection for influenza vaccines" was proposed and the mandate granted by the CHMP to bring together experts from academia, EMA, EDQM and Industry. The workshop was initially scheduled for April. Due to some difficulties with travel arrangements (ash cloud), the meeting was postponed and split into two events.

The first event took place in April, as a Teleconference meeting, to address the technical aspects of the serology assays and their variability.

The second part of the work was scheduled for the end of June, under the format of a workshop to i) further discuss the serology assay aspects, and particularly the possibility to develop more reliable and relevant assays, and ii) discuss the clinical aspects of the serology testing in an attempt to identify the best clinical correlates in terms of seroconversion and seroprotection and the various context of seasonal epidemic or pandemic.

The overall objective of the meeting was to review and put together all the available pieces of information and clinical evidence as well as identifying the missing information and possible development to be considered so as to further improve the assessment of the flu vaccination strategy.

This meeting was not aimed at deciding on the revision of the "CHMP criteria", but rather to provide the scientific basis to the CHMP on the need, or not, for re-considering those criteria, or improve/add some specific considerations essentially on the age of the population to be vaccinated (namely paediatric, elderly, pregnant women) and the pandemic, prepandemic or seasonal contexts.

Four sessions were organised for this scientific workshop (see agenda in appendix 1)

⇒ Session 1: on the correlate of protection, what we know from the past experience

⇒ Session 2: on the assays and their technical difficulties. This section should be considered as a complement to the April meeting, organised via teleconference

⇒ Session 3: on the other methods and targets to be considered to assess and monitor the immune response and/or protection

⇒ Session 4 dedicated to prospective discussions on future investigations and research to be implemented.

This report will thus be organised into two parts dealing with the serology assay (Dr. J. Wood rapporteur) and the clinical correlates (Prof. M. Granstrom, rapporteur) respectively.

3. Serological assays (Rapporteur - J. Wood)

3.1. Report of a teleconference held on 21 April 2010 - J. Wood

An EMA ad-hoc teleconference on collaborative studies for influenza vaccine serology and the serology retesting exercise was held on 21 April 2010 and was summarised in the document EMA/416763/2010. Dr Wood summarised the main messages from the teleconference (see report of the teleconference meeting in annex 2). The following report will only consider the additional data and further discussions on these topics.

3.2. Overview of collaborative studies - E. Terao and A. Daas

Drs Terao and Daas presented the results of an EU collaborative study for the standardisation of Haemagglutination-Inhibition (HI) and Single Radial Haemolysis (SRH) serological assays for seasonal influenza vaccines: impact on compliance with the CHMP criteria (BSP063). This study had previously been presented at the 21 April 2010 teleconference. The authors reiterated their comments on the inherent variability of those tests and recommendations to further reduce the variability were made and particularly i) to use standardised procedures; ii) to monitor technical proficiency; iii) to use SRH in preference to HI for influenza B assays; iv) to use the Beyer correction for pre-vaccination antibody titres; v) to issue guidelines on selection of clinical trial serum panels.

The authors commented also on some shortcomings of the protective rate criterion of the CHMP requirements. They indeed stressed that, with its current design and due to the variability of the test applied, the criterion can be met even before vaccine is administered. Thus for seasonal vaccines, where only one out of the three CHMP criteria must be met, a vaccine may comply with CHMP requirements by meeting the protective rate criterion even before the vaccine is administered. In addition, the protective rate criterion is more subject to systematic difference between laboratories than are other criteria.

3.3. Overview of collaborative studies – J. Wood

Dr. Wood presented the results of WHO collaborative studies on influenza serology. These studies had previously been presented at the 21 April 2010 teleconference. Of relevance to the meeting was the preparation of a candidate International Standard for antibody to pandemic H1N1 virus (code 09/194). Dr Wood reminded the meeting that during the April teleconference it had been acknowledged that the use of an "antibody standard" significantly reduced inter and intra-lab variability.

3.4. Overview of outcome of retesting exercise – J. Wood and R. Wagner

Drs Wood and Wagner summarised the results of the exercise to retest sera from H1N1 pandemic vaccine trials. The sera were tested at NIBSC for HI antibody and at PEI for Virus Neutralisation (VN) antibody. These studies had previously been presented at the 21 April 2010 teleconference. Dr Wagner also commented on future studies planned at PEI to evaluate the contribution to immunity of antibody to neuraminidase (NA); the contribution of different antibody classes to serology assays; the effects of different VN procedures in an attempt to reduce variability of the results induced by different testing protocols.

3.5. Overview of current assays and areas for consideration to improve currently used assays

3.5.1. HI – J. Wood

Dr. Wood presented an overview of the HI assay including the basic principles; a technical description; some pros and cons of the assay and some possibilities for improvement. The assay depends on antibody inhibiting binding between influenza virus haemagglutinin (HA) and sialic acid receptors on the surface of erythrocytes (usually turkey). It has been in use for influenza antibody assays for over 60 years and is the accepted 'gold standard', being accepted by regulatory agencies throughout the world. It is technically simple, easy to automate and there are correlates of protective immunity established for seasonal influenza. However the HI assay is insensitive for antibody to influenza B viruses; needs horse erythrocytes for H5 and H7 viruses of avian origin; and is not very reproducible between different laboratories. It may be possible to improve the HI assay by use of common protocols and reagents amongst laboratories and by use of antibody standards (see above). In addition, because the HI assay has recognised deficiencies (described above) and its functional activity may not entirely reflect biological relevance to protective immunity, it is desirable to supplement HI assay data by use of more functional assays such as VN. There is a need to better understand the HI correlates of protective immunity especially in the context of a pandemic influenza vaccination campaign.

3.5.2. SRH - E. Montomoli

Professor Montomoli presented an overview of the SRH assay. The assay is set up by preparing agarose gels containing influenza virus adsorbed to sheep erythrocytes, in the presence of guinea pig complement. Sera containing antibody to influenza virus are introduced into wells in the agarose and the antibody diffuses into the agarose to initiate zones of complement-mediated lysis of erythrocytes. The size of the haemolysis zone (SRH) is proportional to the amount of anti-influenza antibody which under normal assay conditions is exclusively anti HA antibody. The SRH assay is accepted by the CHMP as a serological endpoint for clinical trials of both seasonal and pandemic influenza vaccines and an SRH area of 25mm2 is considered to indicate protective immunity (as shown by vaccine efficacy trials, see part 2 of this report). Professor Montomoli described the extensive validation studies (in accordance with the current ICH validation protocols) performed on SRH including specificity, repeatability, precision, linearity and robustness. The assay has been established, in terms of its analytical performances for seasonal influenza as well as for H5N1 and pandemic H1N1 viruses. Some critical features to control and improve the SRH assay are the type of erythrocyte; the origin and passage history of the antigen, the type of complement and the suitability of serum controls. A distinct advantage of SRH assay is the ability to use either live or inactivated virus.

3.5.3. Virus neutralisation (VN or MN) - K. Höschler

Dr. Höschler gave an overview of the VN assay. The assay is capable of detecting antibodies which neutralise both influenza infection and virus release from cells by interaction of antibody with virus HA and NA proteins. There are two main "VN protocols", which differentiate essentially by the incubation time (contact time between viruses and cells) and the resulting read out. In general VN assays with short incubation times (1-2 days) use NP protein detection by ELISA; luciferase or GFP activity, whereas longer incubation VN times (≥ 4 days) involve haemagglutination titration of supernatant;

cytopathic effect or plaque reduction in cell monolayer. These differences in the test format may be responsible for inter-lab variable results as the antibody response measured may be of different nature (see below in the clinical correlate section).

Validation and standardisation of VN assays including precision; use of cell and virus controls and titration of the virus infective dose was described. The shorter incubation format of VN assays is particularly suitable for automation and is more reproducible than the longer incubation assays probably due to cells becoming detached from the cell culture plates during long incubation. VN assays are generally more sensitive than HI but require live virus which can be a problem when working with highly pathogenic viruses. Dr. Höschler identified gaps in current knowledge and understanding of the assay performances, and suggested ways to improve the assays. It is important to identify targets for virus neutralisation and the epitopes important in protection. In this regard, phage display studies are in progress. Antibody to virus NA is important in preventing replicated viruses to release from cells and the role in protective immunity needs to be better understood by use of suitable VN assays, such as NA pseudotype release assays in conjunction with the newly developed neuraminidase-inhibition (NI) assays. Similarly, assays to detect antibody to M2e protein; the conserved regions of HA (eg HA2) and Th1 or Th2 directed immune responses will lead to a better understanding of molecular substrate(s)/target(s) of the protective immunity.

During discussion it was suggested that defective interfering influenza particles could be a source of difference in VN results between laboratories.

3.5.4. Measuring cell-mediated immunity - E. Soethout

Dr. Soethout reminded the group that measurement of cell mediated immune (CMI) responses is encouraged by EU regulatory authorities for certain vaccines and he provided an insight into the mechanisms involved in the humoral and innate immune responses to influenza. T cell responses are often cross reactive because they target conserved epitopes. The importance of CMI in protection against influenza is still being elucidated but this understanding is complicated by the variety of T cell assays being used.

Dr. Soethout stressed that it was important to validate the assays (specificity, accuracy, linearity, range, sensitivity, and repeatability, robustness) to ensure better agreement between laboratories. Following training at Dr Soethout's laboratory, four laboratories compared the Granzyme B and Cytokine assays using frozen PBMCs from multiple donors. In general intra-laboratory repeatability and inter-laboratory reproducibility CVs were below 50% for both assays. However the Granzyme B assay offered greater robustness overall, thus offering greater powers of discrimination between positive and negative outcomes. An adaptation of CMI cytokine assays for pandemic viruses using inactivated virus and CMI assays using flow cytometry to allow phenotyping of responses were described.

3.5.5. Evaluation of neuraminidase content in vaccines and immune response – M. Eichelberger

Dr. Eichelberger gave a timely reminder about the importance of both presence of NA as antigen in influenza vaccines and antibody to NA in immunity to infection. The traditional thiobarbituric acid (TBA) method to evaluate anti-NA antibody is both laborious and uses large quantities of dangerous chemicals so it is not often used. Dr. Eichelberger has developed two new assays: a miniaturised TBA assay and an Enzyme-linked lectin assay (ELLA), both assays can be performed in microtitre plates and

are safer than traditional TBA assays. Reassortant viruses with irrelevant HAs were used to produce reagents necessary to validating both assays. Both assays are also capable of analysing the specificity of antibody binding to NA and thus have the potential to examine NA antigenic drift and measuring the antibody to NA induced by vaccines. The robustness of both assays in different laboratories needs evaluating. Dr. Eichelberger is also developing an assay for the NA content of influenza vaccines.

3.6. General summary and conclusions of the Rapporteur

The rapporteur summarised the conclusions from the discussion and identified actions for the future. The conclusions and actions listed below were agreed at the meeting.

3.6.1. Serology assay

a) The conventional HI, VN, SRH serology assays are the only assays currently available for routine and wide scale assessment of influenza vaccine immunogenicity and should be retained in the battery of tests for acceptance criteria for flu vaccines.

⇒ HI data correlate with protective immunity in many studies, whereas fewer studies have confirmed a correlation between SRH data and protective immunity.

- The data were generated many years ago and new studies are needed, using PCR-confirmed infection, to further confirm the validity of the criteria applied for this test

⇒ VN assays offer the most relevant and sensitive measure of post-vaccination antibody but need clinical validation by field studies before they can be considered by regulatory authorities as additional acceptance criteria

- This should include the different formats of VN assays

⇒ Each assay measures different, but overlapping populations of antibody, mainly to HA.

⇒ Several collaborative studies have demonstrated that the assays are reproducible within but variable between laboratories. This interlab assay variability affects compliance with CHMP criteria so that for any given clinical trial, one laboratory could obtain serology data which comply, whereas another laboratory could obtain data which fail. In addition the assay variability could affect the ability to compare different vaccines and the ability to develop meaningful correlates of immunity.

⇒ Further assay standardisation is needed to reduce the variability. The following actions should be encouraged:

- Agreement on the key parameters of each assay to control; i.e. agreed SOPs
- Proficiency studies
- Validation of assays using ICH guidelines
- Use of the international antibody standards, regional standards or panels of sera. The feasibility to produce and use antibody standards for seasonal influenza vaccines needs to be explored
- Possibly use central laboratories for retesting subset of samples

⇒ Epitope mapping is important to understand the targets for neutralising antibody to influenza, and identifying potential new seroconversion or seroprotection markers

⇒ Next generation (e.g. M2e) vaccines may need specific assays as they may trigger humoral responses that are not recognised by conventional HI and VN assays. Such vaccines may also need different correlates of protective immunity developing.

b) The role in clinical protection of antibody to NA, as detected by VN assays, needs elucidating

c) New assays for antibody to NA offer promise for antigenic analysis of NA and hence improved vaccine composition; evaluation of immunogenicity of the vaccine NA component; evaluation of NA content of vaccines.

- Robustness of assays in different laboratories needs assessment

d) As a general conclusion, and considering the various difficulties, pitfalls and unknowns highlighted above, there was discussion whether the CHMP criteria for evaluation of influenza vaccine clinical trials should be re-evaluated. It is recommended that the CHMP criteria should be re-established after correlates of protective immunity have been re-evaluated from laboratory-confirmed (PCR or virus isolation) cases of influenza infection. It is acknowledged that this may take several years to achieve so in the interim, the following measures are recommended:

- The CHMP criteria should include HI, SRH and VN assay data
- The relevance of the 'protective rate' CHMP criteria needs to be re-evaluated
- The CHMP criteria should include a statement relating to assay variability and assay standardisation
- The CHMP criteria should include a statement on the relevance of antibody to NA

3.6.2. Cellular Immunology

Measurement of priming and early immunity is important to allow an understanding of vaccine-mediated protection. A variety of different assays to monitor CMI response was described which were essentially proliferation assays; cytokine detection; or multiparameter flow cytometry.

⇒ It is recognised that CMI assays are still in the exploratory phase and there should be attempts to reach consensus on the most appropriate assays before recommending greater use and initiate assay standardisation

⇒ The use of validated CMI assays should be encouraged in vaccine studies, with the view of establishing, if proved useful, new immunological criteria as surrogate for vaccine efficacy.

4. Immune Correlates of Protection (Rapporteur - M. Granström)

4.1. History of Influenza Vaccines and Correlates of Protection leading up to current Licensing Requirements - A.S. Monto

Dr. Monto presented a broad overview of studies aimed at establishing a serological correlate to protection from the 1940s and onward. He in particular highlighted a study by Salk et al. (Am J Hyg. 1945; 42:57-93), showing strong correlation between height of HAI titres and protection. Another highlighted study, showing the same correlation, was by Meiklehohn et al. (Am J Hyg 1952; 55:12-21). A third study was the much cited challenge study by Hobson et al (J Hyg 1972; 70:767-777).

In the first two studies only anti-HA response was measured by HI, while the Hobson study also measured anti-NA response in a subset of sera.

As a summary Dr. Monto showed that the studies conducted in the period 1943-1969 showed a protection against clinical disease (in younger adults) for A strains between 70-90% and for B strains between 35-94% .

Dr. Monto pointed out that different vaccines may have different correlates and may vary by age/risk groups. Presenting the results of 2007-2008 studies of live attenuated influenza vaccine (LAIV) and inactivated trivalent influenza vaccines (TIV) (Monto et al. NEJM 2009; 361:1260) he showed that somewhat different efficacy profiles are achieved when based on case ascertainment obtained by virus isolation on cell culture or by PCR or both and that HAI seroconversion after seasonal LAIV does not correlate well with efficacy. He advocated the use of PCR for case ascertainment rather than serology or virus isolation. His conclusion was therefore to revisit the precise role of antibodies measured by HI in protection using contemporary endpoints (PCR confirmed by cell culture isolation). The methods need to be standardised, first HI assay and VN, and then moving to Cell Mediated Immunity assays.

There was also a need to identify whether correlates are different with the various vaccine formulations and further work for LAIV was needed.

He also recommended using efficacy trials to provide data on protection although placebo controlled studies will be difficult to conduct in the US as General recommendation for flu vaccination in all age groups will be soon available. The situation being different in Europe, data sharing could be the way forward and human challenge studies could be an option.

4.2. Challenges for Licensure of Seasonal and Pandemic Influenza Vaccines" Summary of the ISIRV workshop in March 2010 - L. Haaheim

Dr. Haaheim presented the outcomes of a meeting held in Miami, 1-3 March 2010 on Immune Correlates of Protection against Influenza with 114 participants. 70% of delegates found that the meeting provided excellent/very good information. Bi-annual follow-up workshops were highly recommended.

Dr. Haaheim highlighted the following presentations:

- The presentation made by Dr. Treanor (Treanor, Vaccine 2000;18:899) focused on the lack of correlation of serum antibody to HA after vaccination with LAIV. In constrat, HA IgA >1:64 in nasal wash and serum antibody to NA at >1:4 by HI correlated well with protection rate.

- Dr. Stephenson's lecture on improving immune responses to inactivated vaccines focused on additional antigens of possible importance for protection, e.g. NP, M1, M2e and on other elements of the immune responses to be considered such as CTL, T helper cells and mucosal response.

The strong correlation between HI titres and clinical protection was illustrated by a meta-analysis of 15 clinical trials, presented by Coudeville et al.

The most salient points of the meeting to summarise were that the precise determinant(s) of the serological correlates to clinical protection are not well understood and therefore the exact post-vaccination parameters to be measured are not known. Even the question of what do we want influenza vaccines to protect against remains unanswered. Do we need to protect against infection (sterilising immunity), (severe) illness and hospitalization or fatal outcome. In order to answer these questions it was suggested that regulatory agencies should take proactive initiatives and advise manufacturers to consider measuring, in a prospective approach, other potentially relevant responses in addition to HI/SRH.

4.3. Correlates of protection, paediatric aspects – M. Granstrom

Dr. Granstrom presented a review of the literature with the Cochrane systematic review (Jefferson Lancet 2005; 365:773) as starting point. The study concluded: "Inactivated influenza vaccines in children aged 2 years and younger had similar effects to placebo". The best study was by Hoberman, JAMA 2003;290:1608 and showed over two seasons 69% (95% CI 34;82) and -7% (95% CI -247;67) protective efficacy, respectively. The authors themselves suggested the possibility that in the season with high vaccine efficacy (VE) by chance more primed children had been included than in the low VE period. It may be that TIV works as a booster vaccine but does not prime, at least the paediatric population.

Dr. Granstrom presented another study, not included in the Cochrane review (for having used HAV as placebo) that allowed stratifying children by their pre-vaccination sero-status. This CDC study (Hurwitz, JID 2000;182:1218) showed that all flu cases in the vaccine group were among the prevaccination seronegative (≤5) children. Also in the control group little protection was noted in the seropositive children, raising some important questions regarding the serological assays and the correlates to protection. Dr. Granstrom then reminded that the most widely used serological assay, HI, measures both IgG and IgM and will, in children, lead to an overestimate of the HA immune responses. In constrat, SRH measures only antiHA IgG and should thus to be preferred in paediatric studies. As regards VN, the response measured by this test depends on the format of the assay, in terms of incubation time (see section one). Using the CDC protocol (short-term incubation of 16-18 hours, Harmon, JCM 1988; 26:333) it measures only antibodies to HA.

The most cited correlate of protection (Hobson, J Hyg 1972;70:767) was based on a challenge study performed with strains that were non-pathogenic for humans (nor for ferrets) and thus measuring only a response to infection, mediated by HA, with rises in antibody titres as endpoint.

An US NIH challenge study (Murphy, NEJM 1972;286:1329), performed with a wild-type strain in HI-negative individuals, showed instead a clear correlation between titre levels to NA and clinical outcome, as well as duration and magnitude of virus shedding.

Current vaccines are only controlled for HA content and serological assays used in clinical trials only measure anti-HA. Considering the results of the Murphy trial it could be recommended for development of new flu vaccines for children, that manufacturers also measure the NA content of their vaccines,

using the long-incubation time neutralisation test that measures both anti-HA and anti-NA responses as well as a functional anti-NA assay to be used in the clinical trials.

Conclusions of this review were for vaccine development in children:

- Adjuvanted TIV is needed in priming but dose-finding studies have to be performed for determining the best adjuvant to antigen ratio.
- The primary serological endpoint should be based on the VN assay using the long incubation time (≥5 days);
- The NA content of vaccines should also be monitored; efficacy trials have to be performed and should be used to investigate the best correlates of protection.

4.4. B and T cell related priming – G. Del Giudice

Dr. Del Giudice presented an overview of the current knowledge of priming capacities against influenza virus (vaccine and/or natural exposure), showing that immunological experience to influenza (seasonal) is acquired during childhood and increases with age. However, do antibodies (the ones that are monitored by the currently used assays) tell the whole story? He pointed to the fact that there is a lack of significant cross-reactivity between seasonal and pandemic H1N1, higher sero-positivity to swH1N1 in older people who are also less susceptible to swH1N1 infection and one single dose of swH1N1 vaccine was enough to meet serological criteria for licensure (even without adjuvant). The question whether previous immunological experience with influenza (infection, vaccination or both) could explain the age distribution and the high immunogenicity of one dose was addressed in a ferret study. The ferrets were immunized on D0 with seasonal vaccine with or without MF59 adjuvant and again on D21 with the same seasonal vaccine or with the A/California vaccine with or without MF59 and then challenged on D49. The experiment showed that both immunogenicity and efficacy (protection against the disease) are *enhanced by previous immunization with seasonal* influenza vaccine (better if the seasonal vaccine was adjuvanted with MF59). The enhanced immunogenicity and efficacy did not seem to be due to cross-reactive antibodies, at least not as measured by HI and VN, suggesting that it could be due to memory low affinity B cells or memory CD4+ cells. He then showed that different techniques can be used to identify these cells. Measurement of specific B and T cells can represent a very good alternative to antibody quantitation to identify priming events in immunologically naive individuals. However, despite the impressive technological progress made in the past few years in this field, measurement of B- and T-cell responses still faces several hurdles: standardization of cell preparation, timing of blood draw, reproducibility/inter-laboratory comparability, volumes of blood (an issue in paediatric trials) and costs.

4.5. From 1918 to the present: the usefulness of the human viral challenge model - J. Oxford

Dr. Oxford presented the history of human challenge studies and current studies ongoing in his laboratory. Human challenge studies are demanding as they have to be performed in isolation facilities of high quality and operating standard but he also showed that there are many relevant applications, e.g. studies in diagnostics, virus characterization, proof of concept of novel vaccines, novel antiviral drugs and disease aetiology and modelling. Dr. Oxford then showed an influenza study with 17 volunteers, median age 25 years, who were inoculated and then reviewed for symptoms twice daily and had nasal lavage for viral shedding (cell culture assay). PBMC were collected at D-1, D7 and D28.

The T cells were separated and stimulated using a pan-influenza genome peptide stimulation matrix and ELISPOT interferon-γ assay. The study showed that 15/17 volunteers, seronegative for H3N2, showed baseline T cell responses to internal M protein and that the viral load in nasal wash was proportionate to the number of pre-existing T cells. Infection by nasal route of influenza A induced marked T cell responses by 3 to 4 fold to all proteins in 12 volunteers on day 7 after infection. The conclusion was that current serological assays do not identify all primed individuals and also that human challenge studies could be used to answer many basic questions in immune response, clinical protection and correlates of protection.

4.6. Do we need different immune correlates for seasonal and pandemic scenario? - M. Granstrom

Starting by the answer to the question: No, but there may be different pandemic scenarios, Dr. Granstrom noted that the basic issue to elucidate is whether protection against disease is based on type-specific antigens, namely HA or on NA. This could be done by cross-protection studies in ferrets with adjuvanted H1N1 and H5N1 but also by measuring antibodies to NA.

A third possibility was to look at VN data from H5N1 studies with the standard protocol of ≥5 days of culture, which will also measure antibodies to NA while seropositivity to H5 revealed to be low in the HI assay. The only manufacturer (GSK) that used the long incubation time VN format for H5N1 (7 days culture) encountered problems with this test applied to samples from subject vaccinated with swH1N1. An explanation can be found in the following studies. The first by Li (J. Clin. Virol. 2009:46:325) that showed H5N1 to induce antigen expression in a large number of cell lines while swH1N1 and seasonal H1N1 did so only in a human cell line. The second was the Hurwitz study (JID 2000;182:1218) which raised the question on why the seronegative children in the vaccine group were not protected by non-adjuvanted TIV and the answer to this question was given in the recent study (Khurana Sci Trans Med 2010;2:15) showing that 2 doses of 15 µg H5N1 vaccine do not prime to NA. It is reminded that the question why seropositive children in the control group were not protected has been answered by earlier studies (Davenport, Arch Environ Health 1970;21:307), showing that primary infection does not prime to NA but repeated exposure is needed. But then why did one-dose of non-adjuvanted pandemic vaccines seem to prime adults as measured by HI?

The answer was offered by the VN data for H5N1 from GSK (using the long incubation time) showing a more correct rate of seropositives with 30% seropositive in children 3-8 years, 45% seropositive in adults 18-60 years and 90% seropositive in adults >60 years of age. Since GSK starting dilution was high (1:20) primed individuals with lower titres were not identified unless given a "booster dose" (to N1) of seasonal Fluarix, when the true rate of primed individual could be identified at 52%, 80% and 98% respectively. The responses from non-adjuvanted pandemic vaccines, which had been considered as priming responses, were in fact booster responses in an extensively primed population. The conclusion would thus be that if a pandemic is caused by a N1 and N2 strain, a "seasonal" pandemic strategy could be used (as the population is "seasonally" sensitised to N1 and N2 strains) but for strains with other NA types with potential to cause human disease (e.g. H7N7), where the population is likely to be naïve and have no protection, a mock-up vaccine strategy is needed.

4.7. General summary of the Rapporteur

Dr. Haaheim raised the fundamental question of the exact expectation in "protective effect" for influenza vaccines. In acute infections it is the general view that vaccines are to protect against clinical disease while in persistent or latent form of infections protection against infection is sought.

Influenza has been claimed to be an exception from this general rule since it has a short incubation period, which would require protection against infection (sterilising immunity); protection that may be provided, according to the studies quoted above, by the anti-HA as detected by HI tests. This strategy is however difficult to fully reconcile when considering the efficacy of the antiviral agents (neuraminidase inhibitors) when given in post-exposure prophylaxis to household contacts – and in some cases without treating the index case. Neuraminidase inhibitors provided to the family members an effective protection against disease (but who were surely infected). As such, not only antiHA response but also antiNA response should be considered in the protection strategy against infleunza.

Dr. Monto and Dr. Haaheim showed studies confirming that antibodies to HA measured by HI correlate well with protection against disease in adult and elderly populations. These studies were however conducted in adults that were more than likely primed individuals, and usually only the response by HI (seroconversion) was measured. It is however well-known from other vaccine fields that correlates to protection cannot be established in primed individuals since the booster responses of an individual is determined by his/her genetic set-up, being a low-responder, a high-responder or a majority in between, and thus measuring the response to one antigen could in fact mirror the equally high response to another unmeasured antigen.

The correlate of protection, as mainly measured by HI, comes from the large challenge studies conducted in the UK with temperature sensitive mutants, called attenuated strains. These strains had been rendered non-pathogenic but could still infect, for which reason a correlation to HA (sterilising immunity) but not NA (protection against clinical disease) was found. A challenge study from the US in HI negative volunteers showed a direct correlation between the level of antibodies to NA and clinical outcome of the infection caused by a wild-type strain. This study clearly raises the question of the usefulness of considering NA response in the "protective effect" of a flu vaccine. Human challenge studies can be conducted even today, as elegantly shown by Dr. Oxford, and the availability of neuraminidase inhibitors reduces the risk to the volunteers.

Correlates to protection can be established also in paediatric studies and the planned studies with adjuvanted TIV should be used to do so. An adequate correlate to protection has a major impact on all parts of the influenza field: vaccine composition, choice of serological assays and last but not least knowledge of pre-existing priming in the population and thus pandemic preparedness plans. The issue deserves attention and particularly the respective role of anti-HA and anti-NA antibodies in the immune response (prime and boost) and the corresponding clinical protection against the agent and/or the disease. This is of importance for the future strategy in preparation to a new pandemic phase, which may be caused either by a new NA type that has never been circulating or a NA type that has been circulating as seasonal strains.

4.8. Summary and conclusions on correlates to protection -M. Granström

The correlates of immune protection were mainly based on human challenge studies conducted at the time of the Hong Kong pandemic. The most often quoted study, by Hobson, (J Hyg 1972; 70:767) used so-called attenuated temperature sensitive strains rendered non-pathogenic for ferrets and humans. The endpoint of the trials with these mutants was titre rises by HI, i.e. infection, and therefore showed a correlation to antibodies against the haemagglutinin (HA).

Vaccines against acute infections are to protect against clinical disease while those against diseases with a persistent or chronic phase will also have to protect against infection. The hypothesis that influenza would be an exception to this general rule and would only need sterilising immunity was based on its short incubation period. However, it is noteworthy that neuraminidase (NA) inhibitors when given in post-exposure prophylaxis have later shown that they could protect against clinical disease without protection against infection.

In support of this, another human challenge study, performed by the US NIH (Murphy, NEJM 1972; 286: 1329), used a wild-type strain and the volunteers were screened to be HI sero-negatives. In these individuals both the clinical presentation of the disease, the amount of virus excreted and the length of excretion was directly correlated to the level of pre-existing antibodies to NA.

The consequence of focusing mainly on the sterilising immunity (seroconversion to HA) has lead to measuring only antibodies to HA during the past decades (HI and SRH). The neutralisation test (VN), with a sufficiently long incubation time (> 5 days) would measure antibodies to all structures needed for protection. However, by decreasing the incubation period for the test to 16-18h (Harmon, JCM 1988; 26:333) the assay was corrupted since these culture conditions will only measure the immune response to the first cycles of the infection, mediated by HA, but not to later stages, mediated by NA. This assay with the short incubation period is used by all agencies and all manufacturers with one exception: GSK used the regular NT for H5N1 and while all short-incubation assays show 1-2% pre-vaccination sero-positives (to H5) GSK reports some 80% primed adults (to N1). The high rate of primed individuals in the population also explains the mild course and rapid resolution of the current swH1N1 pandemic.

The need to induce protective antibodies to NA to get a vaccine protective against the disease entails a number of changes:

1. Influenza vaccines contain currently standardise amounts of HA but need to be standardized also for NA content. This could be achieved by using an animal model where the antibody response to the major antigens (HA and NA) has to be at least equal to those elicited by a reference vaccine that has been shown protective in an efficacy trial
2. Antibody responses to NA by a functional assay needs to be assessed separately in clinical trials
3. The VN test with long incubation time (> 5 days) needs to replace the short-term culture version so as to identify the whole immune response in priming, and particularly for the paediatric population

4. Responses to vaccination need to be evaluated for pre-vaccination sero-negatives and sero-positives separately and these individuals can only be identified by use of a functional assay (anti-NA and/or regular NT) as primary end-point

5. A booster dose needs to be given in all primary vaccination strategy in order to ensure priming to NA

5. Attachments

5.1. Agenda of the Workshop held on 29th of June 2010

5.2. List of Participants of the Worksop held on 29th June 2010

5.3. Declarations of Interest – Appendix to List of participants

5.4. Report of the Ad-hoc teleconference on Serology retesting exercise and collaborative studies for Influenza vaccines held 21st April 2010

5.1 Agenda of the Workshop held on 29th of June 2010

29 June 2010
EMA/732806/2010
Human Medicines Development and Evaluation

Scientific Workshop on Serology Assays and Correlates of Protection for Influenza Vaccines
29 - 30 June 2010

Agenda
Room 2D

Chair J-H Trouvin
Rapporteur: J Wood (Assays)
Rapporteur: M Granstrom (Immune Correlates)

		29 June
	Registration	9.00
1	**Introduction and welcome** Objectives of the meeting: J-H Trouvin Declaration of Interests	9.40
2	**Immune Correlates of Protection: Overview of current status and identification of limitations**	
2.1	History of Influenza Vaccines and Correlates of Protection leading up to current Licensing Requirements: AS Monto	10.00
2.2	Immune Correlates of Protection Against Influenza: Challenges for Licensure of Seasonal and Pandemic Influenza Vaccines" Summary of the isirv workshop in March 2010: L Haaheim	10.30
2.3	Correlates of protection, paediatric aspects: M Granstrom	11.00
	Coffee	11.30
2.4	B and T cell related priming: G Del Giudice	12.00
	Discussion on 2.1 to 2.4	12.30
	Lunch	13.00
3.	**Serological Assays for Influenza Vaccines**	
3.1	Report of teleconference held on 21 April 2010	
3.1.1	Overview of collaborative studies (E Terao, A Daas and J Wood)	14.00

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3.1.2	Overview of outcome of retesting exercise (NIBSC/PEI) *Anonomysed data and main messages*	14.30
3.2	Overview of current assays and areas for consideration to improve currently used assays (HI, SRH, MN) - HI: J Wood - SRH: E Montomoli - Microneutralisation (MN): K Hoschler	 15.10 15.35 15.55
	Coffee	16.15
4.	**Other methods for investigation as potential assays and other targets**	
4.1	Measuring cell mediated immunity: E. Soethout	16.35
4.2	Evaluation of Neuraminidase content in vaccines and immune response: M Eichelberger	16.55
	Discussion on sections 3 and 4	17.15
	Close of day 1	18.15
		30 June
5	**Areas for consideration for future investigation/research**	
5.1	Current Activities at DG Research: C Desaintes	9.15
5.2	From 1918 to the present: the usefulness of the human viral challenge model: J. Oxford	9.45
5.3	Do we need different immune correlates for seasonal and pandemic scenario? M Granstrom	10.15
5.4	Technical aspects of assay methods: The way forward – topics for discussion J. Wood	10.45
	Coffee	11.15
	Discussion 5.1 to 5.4	11.30
6	**Conclusion (Chair)**	12.30
	Close	12.45

5.2 List of Participants of the Worksop held on 29th June 2010

Scientific Workshop on Serology Assays and correlates of protection for Influenza Vaccines
29 – 30 June 2010

List of Participants

Participants:

Name	Affiliation
Claudia Alfonso	World Health Organisation
Barbara Capecchi	Novartis Vaccines
Laura Compitelli	Instituto Superiore di Sanita
Francois Cano	French Health Products Safety Agency (AFFSAPS)
Arnold Daas	European Directorate for the Quality of Medicines & HealthCare (EDQM)
Martine Denis	Sanofi Pasteur
Roland Dobbelaer	Federal Agency for Medicines and Health Products Belgium
Daniel Brasseur	Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten - Belgium
Christian Desaintes	European Commission
Jeanne-Marie Devaster	GSK Bio
Othmar Engelhardt	National Institute for Biological Standards and Control (NIBSC)
Constanze Goepfert	Paul-Ehrlich Institute – Germany (PEI)
Marta Granstrom	Karolinska University – Hospital Solna
Giuseppe Del Giudice	Novartis
Lars R. Haaheim	University of Bergen
Anne Katrin Hilbert	Novartis Vaccines
Steve Hildreth	Sanofi Pasteur
Marcel H.N. Hoefnagel	National Institute of Public Health – NL
Katja Hoschler	Health Protection Agency
Keith Howard	Baxter
Otfried Kistner	Baxter
Urban Lundberg	GSK Bio
Jose Melero	Institudo de Salud Carlos III
Arnold S Monto	School of Public Health, University of Michigan, USA
Emanuele Montomoli	University of Siena
Mariska Mulder	Abott Biologicals
John Oxford	St. Bartholomew's Hospital – London
Michael Pfleiderer	Paul-Ehrlich Institute – Germany (PEI)
Mair Powell	Medicines and Healthcare products Regulatory Agency – UK (MHRA)
James Robertson	National Institute for Biological Standards and Control (NIBSC)
Sandrine Samson	Sanofi Pasteur MSD
Corina Schmidt	GSK Bio
Brunhilde Schweiger	Robert Koch Institute
Ernst C. Soethout	Nederlands Vaccin Instituut
Nigel J. Temperton	University of Kent
Eriko Terao	European Directorate for the Quality of Medicines & HealthCare (EDQM)

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Rigmor Thorstensson	Sweedish Institute for Infectious Disease Control
Jean-Hugues Trouvin	French Health Products Safety Agency (AFFSAPS)
Jaap Venema	Abott Biologicals
Mireille Vossen	Abott Biologicals
Ralf Wagner	Paul-Ehrlich Institute – Germany (PEI)
Bettie Woordouw	College ter Beoordeling van Geneesmiddelen NETHERLANDS
John Wood	National Institute for Biological Standards and Control (NIBSC)

Participating via teleconference

Rebecca Cox	University of Bergen
Karl-Heinz Buccheit	European Directorate for the Quality of Medicines & HealthCare (EDQM)
Maryna Eichelberger	Food and Drug Administration
Kari Johansen	European Centre for Disease Prevention and Control (ECDC)

EMA secretariat:
Richardson Peter; Gate Nick; Pedone Elisa; Jekerle Veronika; Trullas Jimeno Ana; Tiitso Klara; Martino Carla; Cavaleri Marco; Ehmann Falk; Ruepp Robin; Olivier Sophie;

5.3 Declarations of Interest – Appendix to List of participants

Scientific Workshop on Serology Assays and correlates of protection for Influenza Vaccines

Attachment to the List of Participants

Declaration of Interests

In addition to the industry representatives, the following declarations of interests were made:

1. Professor A.S. Monto (University of Michigan) mentioned that he is consultant with Biocryst and in the last year was a consultant for Baxter. He is a member of advisory boards for Roche, GSK and Novartis. He is a principal investigator for Sanofi Pasteur.

2. Professor E. Montomoli (University of Siena) mentioned that he is an investigator (not principal) for Novartis, Baxter and Sanofi-Pasteur

3. Professor R. Thorstensson (Swedish Institute for Infectious Diseases) mentioned that more than one year ago but less then five years ago she was a consultant for Eurocine.

4. Professor R. Cox (University of Bergen) mentioned that she is a consultant to Novartis and is an investigator (not principal) with Crucell and GSK, previously in the last 5 years she has been an investigator (not principal) for Sanofi Pasteur.

5. Professor J. Oxford (St.Bartholomew's Hospital – London) mentioned that he is also Director of Retroscreen and is a member of Advisory boards for GSK, Novartis, Medimmune, Roche and Baxter.

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5.4 Report of the Ad-hoc teleconference on Serology retesting exercise and collaborative studies for Influenza vaccines held 21st April 2010

Ad Hoc Teleconference on Serology retesting exercise and collaborative studies for Influenza Vaccines

Doc.Ref.: EMA/732806/2010

21 April 2010 – 2:00-6:00 pm (UK time)

Chair J-H Trouvin

Rapporteur: J Wood

The ad hoc teleconference was held in lieu of a postponed Workshop on Scientific Aspects of Serology Assays for Influenza Vaccines (postponed due to flight cancellations in Europe). The teleconference reviewed the outcome of influenza vaccine serology collaborative studies and the outcome of the retesting exercise. The telecon was held with European Medicines Agency Task Force (ETF), Vaccine Working Party (VWP) and Biologics Working Party (BWP) experts and EDQM experts.

EDQM collaborative studies

These studies were presented by Karl-Heinz Buchheit, Arnold Daas and Eriko Terao, EDQM. The collaborative studies (BSP063) were conducted within the framework of the Biological Standardisation Programme of EDQM and were organised into two phases: Phase 1 Impact of technical differences on compliance with CHMP criteria, project leader J Wood; Phase 2 Improvement of reproducibility of clinical trial serology and impact on compliance with CHMP criteria, project leaders J Wood and E Montomoli (University of Siena). Both studies included a mixture of vaccine manufacturers and public laboratories and examined panels of sera from influenza vaccine trials. In Phase 1 only the Haemagglutination-Inhibition (HI) assay was examined but in Phase 2 both HI and Single-Radial Haemolysis (SRH) was used. The study designs allowed for each serum panel to be tested by three laboratories in Phase 1; four laboratories in Phase 2 (HI) and three laboratories in Phase 2 (SRH). In Phase 2, one central laboratory tested all serum panels by the HI and another by SRH. The main conclusions are as follows:

- In both Phase 1 and 2 studies there were differences in HI results from different laboratories which affected compliance to the CHMP criteria. The efforts to improve standardisation of assays in Phase 2 did not affect the outcome.
- In Phase 2, SRH data were not as variable as HI data but there was still variability in meeting the CHMP criteria, in different laboratories
- The use of a sheep antiserum as a standard did not improve the reproducibility of either HI or SRH data
- The use of a linear regression (Beyer correction) to correct for pre-vaccination status of subjects successfully reduced systematic bias in Phase 1 study but not in Phase 2. The application of this correction factor is still uncertain.
- Although better assay harmonisation has been attempted since the very beginning of this two-phase collaborative study, by sharing SOPs, using common key reagents and reaching agreement on key assay features, the results clearly showed that there

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remain local factors (e.g. source of erythrocytes, interpretation of the scoring procedures) which are difficult to control.

WHO collaborative studies

These studies were presented by John Wood, NIBSC. Three studies were described: an H3N2 study in 2007, an H5N1 study in 2009 and a pandemic H1N1 study in 2009-10. Each of the studies involved a mixture of vaccine manufacturers and public laboratories and examined sera from influenza vaccine trials and convalescent subjects (H1N1pdm study only). Both HI and Virus Neutralisation (VN) assays (local methods) were examined and in each study the impact of a human antibody standard was evaluated. In the H5N1 study a modified HI using horse erythrocytes (hHI) was used. The main conclusions are as follows:

- Reproducibility within laboratories was generally good for both HI and VN assays
- There was significant variability between laboratories for both HI and VN assays; VN was generally more variable than HI
    - HI median GCVs ranged from 31-582% (equivalent to 8-128 fold range)
    - VN median GCVs ranged from 68-383% (equivalent to 31-724 fold range)
- There was no consistent correlation between HI and VN titres
- In the H5N1 study a sheep antibody standard did not reduce variability between laboratories, confirming the conclusion of the EDQM study reported above.
- The use of a human antibody standard to reduce variability between laboratories has been proposed. For that an International Standard for antibody to H5N1 clade 1 (code 07/150) was established by WHO to contain 1000 IU and a candidate International Standard for antibody to H1N1pdm (code 09/194) was evaluated.
    - Using these Standards reduced variability between laboratories by approximately 50%, for both HI and VN.
    - In H5N1 study, this standard 07/150 was not suitable for standardising assays for antibody to clade 2 H5N1.
    - The consensus titres of a candidate International Standard for antibody to H1N1pdm (code 09/194) were HI 1:183; VN 1:516

Outcome of retesting exercise

At a CHMP expert meeting on influenza serology held on 31 July 2009, it was acknowledged that *" it is difficult to assess and compare, not only MN assay data, but also the other serological assays (HI, SRH) from different manufacturers because this assay is not standardised between companies or laboratories that run the tests"*. It was thus concluded *"on the potential benefit of a Central Laboratory to re-test a small subset of samples from clinical trials performed by different manufacturers to further reduce the between-results variability and permit proper conclusions to be drawn in terms of dosage schedule*". (serology meeting report).The subset of sera would be tested centrally by HI at NIBSC and by VN at PEI. The tests would include the new candidate antibody standard 09/194. Each vaccine manufacturer was requested to select a total of 120 sera (low, medium and high titre) from trials in children, adolescents, adults and the elderly. The data from the study were presented by John Wood, NIBSC (HI assay) and Ralf Wagner, PEI (VN assay) and the main conclusions are:

- The data could not be used for evaluating CHMP compliance as they were from a selected subset of sera
- The agreement between HI data from the manufacturers (coded 1-5) and NIBSC varied from good to poor
    - The ratio between NIBSC data and manufacturers' data ranged from 0.4-5.2
- In general manufacturer 5 usually agreed with NIBSC; manufacturer 1 was usually higher than NIBSC; manufacturers 2 and 3 were always higher than NIBSC and

manufacturer 4 was either lower or similar to NIBSC depending on the study population

- The HI titre of 09/194 was 1:167 at NIBSC which was close to the collaborative study consensus; the manufacturers' titres ranged from 1:320-1:720
- There was much better agreement between manufacturers and NIBSC when the data were adjusted by use of 09/194
    - The ratio between NIBSC data and manufacturers' data ranged from 0.2 -1.3 with the mean being 0.6. Ideally this should have been closer to 1.0 but it may have been affected by manufacturers testing 09/194 on a separate occasion to the test sera.
- The agreement between VN data from the manufacturers (coded 1-5 as in the HI analysis) and PEI varied from good to poor
    - The ratio between PEI data and manufacturers' data ranged from 0.2-7.0
    - In general manufacturer 5 usually agreed with PEI; manufacturer 1 was either higher or similar to PEI; manufacturers 2 and 3were always higher than PEI (manufacturer 4did not perform VN assay)
- The VN titre of 09/194 was 1:669 at PEI which was close to the collaborative study consensus
- There was much better agreement between manufacturers and PEI when the data were adjusted by use of 09/194
    - The ratio between PEI data and manufacturers' data ranged from 0.3-5.7, but there were 4 results from comparison of manufacturer 1 data that were outliers at >4.0. The reason for this is not known. The remainder (86%) were in the range 0.4-2.1 with the mean being 1.0

Overall conclusions and implications

- `The EDQM study demonstrated that better HI or SRH assay harmonisation did not reduce variability between laboratories. The impact of further standardisation (e.g. harmonisation of scoring procedures) on the reduction of this residual but large variability is to be proved.
    - There are no data on VN assay harmonisation
- A sheep antibody did not function as an antibody standard to reduce variability
    - Other animal sera could be evaluated eg ferret
- From the collaborative studies, it was concluded that the correlation between HI and VN may not be achieved, not only due to the variability of the two assays but also due to the fact that VN is measuring an immune response (antibodies against neutralising epitopes on HA and possibly NA) which is qualitatively different from the response measured by HI (antibody against epitopes adjacent to receptor binding site of HA). The two assays should be viewed as complementary tests and not as alternative.
- Human antibody preparations used as standards consistently reduced HI and VN assay variability between laboratories and allowed better comparison of vaccine trial data. This approach seems to be the most promising for further reduction of the interlab variability. However this is subject to several logistical difficulties and solution have to be elaborated:
    - For seasonal vaccine trials, an antibody standard is probably needed for each strain in the vaccine and as it took 3 months to prepare the H1N1pdm International Standard, this approach is probably not feasible
    - Sera from recipients of an adjuvanted vaccine may be more cross-reactive and should be evaluated as a longer term antibody standard
    - A short term antibody standard could be prepared each year by pooling sera from the first available vaccine trial (low, medium and high titre pools) and rapidly distributing the pools to EU serology laboratories. The serum pools could serve as antibody standards for vaccine serology.
- The retesting exercise was valuable in allowing better comparison of data from vaccine manufacturers and was even better when an antibody standard was used.

- Due to the complexity of the exercise, and despite the interesting outcome, the group concluded that it may be difficult to sustain this for seasonal vaccine trials. Clearly, other methods or approaches, and particularly the use of an antibody standard preparation should be envisaged to allow proper interpretation of the results provided by the different manufacturers.