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Enhancing the reproducibility of serological methods used to evaluate immunogenicity of pandemic H1N1 influenza vaccines—An effective EU regulatory approach

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ABSTRACT

Haemagglutination-inhibition (HI) and virus neutralisation (VN) assays are routinely applied to evaluate influenza vaccine immunogenicity for regulatory approval. Despite their frequent use both assays are currently only poorly standardised causing considerable inter-laboratory variation of serological results that is particularly evident for pandemic influenza vaccines. The present study was conducted in association with the European Medicines Agency (EMA) to directly compare assay variability between vaccine manufacturer's and European regulatory agency's laboratories in an influenza pandemic scenario. To this end, a defined subset of H1N1pdm clinical trial sera from all manufacturers that had applied at EMA for approval of pandemic H1N1 vaccines were re-tested by the National Institute for Biological Standards and Control (for HI) and the Paul Ehrlich Institute (for VN). Comparative analysis of test results determined for almost 2000 serum samples revealed a marked inter-laboratory variation for HI titres (up to 5.8-fold) and even more for neutralisation titres (up to 7.0-fold). When the absolute titres were adjusted relative to the calibrated International Antibody Standard 09/194 variation was drastically reduced and acceptable agreement of results from different laboratories could be achieved. Hence, inclusion of an appropriate calibrated antibody standard for adjustment of original titres is a powerful tool to substantially increase reproducibility of serological results from different laboratories and to significantly improve regulatory evaluation of influenza vaccine efficacy.

1. Introduction

Within the European Union (EU) regulatory environment, evaluation of influenza vaccine immunogenicity is commonly performed by means of serological assays, mostly haemagglutination-inhibition (HI) and single radial haemolysis (SRH). Both methods basically rely on the ability of the viral haemagglutinin (HA) surface glycoprotein to agglutinate red blood cells (RBC) [1,2]. From the broad database gained over the last decades in clinical studies with either vaccinated or experimentally infected subjects correlates of protection for serum HI and SRH titres have been established that are applied routinely for the evaluation and licensing of seasonal influenza vaccines [3–6].

With the event of influenza outbreaks caused by highly pathogenic H5N1 subtype viruses of avian origin in the late 1990s it was discovered that the conventional HI system was rather inappropriate to assess immunogenicity of newly developed H5N1 pandemic vaccines in clinical evaluation [7]. Primarily this deficiency was attributable to the insensitivity of conventional HI assays using chicken or turkey RBC for the determination of antibody response to avian influenza strains [8,9]. To overcome this incompatibility alternative HI procedures making use of horse RBC were developed that were employed by manufacturers to demonstrate H5N1 vaccine immunogenicity for licensing purposes [7,10,11]. In addition to RBC-based assays, quantitation of serum antibody responses by virus neutralisation (VN) has been implemented as a mandatory regulatory requirement for approval of pandemic influenza vaccines by the European Medicines Agency (EMA) [12]. Although no approved correlate for protection has so far been established for VN titres this assay nevertheless represents a valuable tool to complement HI results. Despite its higher technical complexity VN testing offers a significant added value in that it measures antibodies capable of neutralising viral infectivity and not only those that block the binding of viral HA to RBCs. Irrespective of their frequent use these assays are currently insufficiently standardised and significant inter-laboratory variation has

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been observed in collaborative studies conducted to evaluate the serological assays used to assess the immune response to seasonal and pandemic influenza vaccines [13–16]. It is most likely that the more complex methodology together with the lack of a commonly accepted protocol renders VN testing even more prone to inter-laboratory variability than the HI assay [13].

Among regulatory agencies these difficulties created considerable concern regarding the reproducibility and relevance of immunogenicity testing conducted for approval of novel influenza vaccines. The problem became very evident when facing the diversity of serological data packages submitted to EMA by different manufacturers for licensing of (pre)pandemic H5N1-specific vaccines. This situation prompted extensive discussion at the EMA Committee on Medicinal Products for Human Use (CHMP) and objections were raised about the applicability of the available assays to accurately evaluate immunogenicity of pandemic influenza vaccines. In an expert group meeting held at EMA shortly after the declaration by WHO of the "swine flu" pandemic it was therefore decided that the observed shortcomings in serological data acquisition necessitate a joint approach by the vaccine industry and European control authorities to systemically explore the consistency of immunogenicity determinations during the course of the pandemic H1N1 vaccination campaign. To this end, the expert group adopted a "centralised re-testing" strategy by which a defined subset of sera from H1N1pdm clinical trials already tested at vaccine manufacturer's laboratories was to be re-tested by European control authorities under controlled conditions. The National Institute for Biological Standards and Control (NIBSC, UK) and the Paul-Ehrlich-Institute (PEI, Germany) were designated by EMA to conduct the HI and VN assays, respectively. Here we present the results of this comparative analysis including almost 2000 serum samples from pandemic H1N1 vaccine trials. We observed a considerable overall inter-laboratory variation for HI titres (up to 5.8 fold) and even more for VN titres (up to 7.0 fold). Of prime importance is the finding that adjustment of absolute titres in relation to a calibrated International Standard for serum antibody proved to be a very powerful analytical tool to drastically reduce variability between different laboratories and to allow for a more accurate and reliable regulatory evaluation of pandemic influenza vaccines.

2. Materials and methods

2.1. Serum samples

Test serum samples were provided by four manufacturers from clinical studies conducted for licensing of pandemic H1N1 influenza vaccines. In total sera from trials with five different vaccine products (coded Vac 1–5), including adjuvanted and non-adjuvanted formulations, were tested. The following internal control sera were used for HI and VN testing: (i) normal sheep serum as negative control (Dianova), (ii) serum from influenza A/California/7/2009 (H1N1)pdm infected sheep (NIBSC #09/142) and (iii) human serum pool from convalescent influenza A H1N1pdm cases and recipients of A/California/7/2009 (H1N1)pdm containing vaccines (International Standard for antibody to pandemic H1N1 influenza virus, NIBSC #09/194 [17,18]) as positive controls. The international standard is available from NIBSC, UK [19].

2.2. Serological assays

Serological procedures as conducted at the NIBSC (HI test) and PEI (VN test) are described in detail below. Assay protocols applied at manufacturer's laboratories are to be treated as confidential information not to be disclosed here. However, it is noted that manufacturer's methods are in principle very close to those described hereafter and differ only slightly in certain details of individual procedural steps and materials used.

As antigen source for HI and challenge virus for VN the classical influenza reassortant X179A was used that contains the HA and NA surface glycoproteins of the pandemic H1N1 influenza strain A/California/7/2009 [20].

At central laboratories all serum samples were tested in duplicate HI and VN assays and in cases where the two initial results differed more than twofold, triplicate testing was performed. For analytical purposes, titres below 10 were assigned a value of five.

2.2.1. Haemagglutination-Inhibition (HI) assay

Sera were treated with receptor destroying enzyme (RDE(II), Denka Seiken Co., Ltd.) by diluting one volume of serum in four volumes of RDE and subsequent overnight incubation at 37 °C. RDE was then inactivated for 45 min at 56 °C. Serial two-fold dilutions of RDE-treated sera starting from a 1:2 initial dilution (actual 1:10 serum dilution) in PBS A were prepared in a V-bottom 96 well microtitre plate in a total volume of 25 μl each. Next, 8 haemagglutinating units of influenza reassortant X179A were added to each of the sera dilutions. Back-titrations of virus solution were performed to assure that the correct haemagglutinating HA antigen dose was added. After 1 h 25 μl of 0.7% turkey red blood cells in PBS A were added and the plate was incubated at RT for another 30 min. Following this plates are then read for the highest serum dilution able to inhibit haemagglutination. The reciprocal of this serum dilution represents the HI titre of the respective serum.

2.2.2. Virus neutralization (VN) assay

Neutralisation assay performed at the PEI is a modified version of a previously described procedure [2]. The test was carried out on Madin-Darby-Canine kidney (MDCK) cells seeded in 96-well microtitre plates at a density of 1.5×10^4 cells per well in 100 μl Eagle's Minimal Essential Medium (EMEM) supplemented with 10% foetal calf serum (FCS). Human sera were heat inactivated for 30 min at 56 °C. Starting from a 1:10 initial dilution a twofold serial dilution of inactivated sera was set up in 50 μl virus diluent solution each (EMEM containing 40 mM HEPES, 1% bovine serum albumin, 1 mg/ml TPCK treated trypsin). Next, 100 $TCID_{50}$ of egg grown virus reassortant X179A in 50 μl virus diluent solution were added to each of the sera dilutions, mixed gently and incubated for 1 h at 37 °C, 5% CO_2. Virus back-titration on MDCK cells was carried out in parallel with each VN assay to reassure that the appropriate $TCID_{50}$ was consistently applied in all assays. Following incubation each virus-serum mixture was transferred to one of the MDCK cell monolayer wells (previously washed with virus diluent solution without trypsin) in the microtitre plates and subsequently incubated overnight (18–22 h) at 37 °C, 5% CO_2. As controls, four wells/plate remained untreated (no virus infection, no antiserum addition, cell control) or were infected without addition of antiserum (virus control).

After overnight incubation cells were washed with PBS and fixed with ice-cold 80% acetone (in PBS) for 10 min. Infection of cells was examined by ELISA detection of viral nucleoprotein. For this, cells were washed three times with PBS containing 0.1% Tween20 and treated with a mixture of two monoclonal NP-specific antibodies (MAB8257 and MAB8258, Chemicon) diluted 1:4000, 100 μl/well in PBS with 1% BSA and 0.1% Tween20 for 1 h at RT. Plates were washed three times before the second antibody (goat anti-mouse IgG, HRP conjugated, Kirkegaard&Perry Laboratories) was added (1:2000, 100 μl/well) for 1 h at RT. Plates were again washed as above before 100 μl/well of freshly prepared O-phenylenediamine dihydrochloride substrate solution (10 mg in 20 ml phosphate/citrate buffer containing 0.03% sodium perborate, Sigma–Aldrich) were added. After incubation for 5 min at RT the reaction was stopped by addition of 100 μl/well of 0.5 N sulphuric acid and absorbance (OD)