Journal of Virological Methods 251 (2018) 46–53

Contents lists available at ScienceDirect

Journal of Virological Methods

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

# A latex agglutination assay to quantify the amount of hemagglutinin protein in adjuvanted low-dose influenza monovalent vaccines

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ARTICLE INFO

*Keywords:*
Influenza
Latex beads
Hemagglutinin
Pandemic vaccines

ABSTRACT

To formulate inactivated influenza vaccines, the concentration of hemagglutinin (HA) must be accurately determined. The standard test currently used to measure HA in influenza vaccines is the Single Radial Immunodiffusion (SRID) assay.

We developed a very rapid, simple and sensitive alternative quantitative HA assay, namely the Latex Agglutination Assay (LAA). The LAA uses the Spherotest® technology, which is based on the agglutination of HA-specific immunoglobulin-coated latex beads. The amount of HA in a sample is calculated from the level of bead agglutination by a simple absorbance measurement at 405 nm against a standard curve generated using a monovalent vaccine standard.

In less than 2 hours, tens of samples could be quantified using the LAA as opposed to 2 days for the SRID assay. Ten steps are required to complete an SRID assay as compared to 6 steps for the LAA, from sample preparation through spectrophotometric analysis. Furthermore, the limit of detection of the LAA was found to be approximately 15 ng HA/mL, similar to an ELISA, with the quantification of less than 1.8 µg HA/mL. The quantification limit of the SRID is usually considered to be approximately 5 µg HA/mL.

The development of the assay and a comparison of the titers obtained by SRID and LAA for several monovalent vaccines corresponding to various strains were performed. For A/H5N1 and A/H1N1 monovalent vaccines, the LAA was found to be linear and accurate as compared to the SRID. The precision of the LAA was close to that of the standard test, and good reproducibility from one laboratory to another was observed. Moreover, the LAA enabled HA quantification in AlOOH-adjuvanted and in emulsion-adjuvanted low-dose vaccines as well as unadjuvanted vaccines.

In conclusion, LAA may be useful to rapidly and accurately measure influenza HA protein in monovalent vaccines, especially in those containing less than 5 µg/mL of HA in the presence of an adjuvant.

## 1. Introduction

Influenza viruses are negative stranded RNA viruses of the *Orthomyxoviridae* family. Three types of influenza viruses, influenza A, B and C, are capable of infecting humans, with influenza A and B being the most common circulating types. Influenza A viruses are classified into subtypes based on the antigenic identity of the two major surface glycoproteins on the virion, hemagglutinin (HA) and neuraminidase (NA). Both proteins lead to an antibody responses upon infection and antibodies against HA confer protective immunity (Gomez Lorenzo and Fenton, 2013), while antibodies against NA reduce severity of disease by restricting viral replication (Johansson and Cox, 2011).

Global influenza epidemics emerge seasonally and typically occur during the winter seasons of the northern and southern hemispheres. Seasonal influenza epidemics result annually in 3–5 million cases of severe illness and 250,000–500,000 deaths worldwide (WHO, 2016). The emergence of a pandemic H1N1 strain in 2009 (Neumann et al., 2009) and highly pathogenic avian H5N1 and H7N9 influenza viruses

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http://dx.doi.org/10.1016/j.jviromet.2017.10.011
Received 19 July 2017; Received in revised form 6 October 2017; Accepted 10 October 2017
Available online 10 October 2017
0166-0934/ 

(de Jong et al., 1997; Gao et al., 2013) has reaffirmed that influenza remains a serious global public health concern. Vaccination is considered the most effective strategy to reduce the large morbidity and mortality caused by influenza infection (Poland et al., 2001; Zambon, 1999).

Current seasonal approved-vaccines are composed of either trivalent or quadrivalent mixtures of the most globally-prevalent circulating influenza strains (A/H1, A/H3 and B). Many of these vaccines are produced in embryonated chicken eggs, harvested and processed into either live attenuated, or more commonly, inactivated viral vaccine preparations. The alternatives to egg-based production systems are cell culture-based systems and recombinant influenza antigens. The Food and Drug Administration (FDA) has approved two such vaccines in 2012 and 2013, respectively: Flucelvax (Seqirus), produced in Madin-Darby Canine Kidney (MDCK) cells and Flublok (Protein Sciences Corporation), the first approved vaccine made of recombinant proteins produced in insect cells.

All these vaccines have to be reformulated every year due to the antigenic drift, *ie* mutations in surface glycoproteins, responsible for immune escape. Influenza virus also can undergo antigenic shift due to the reassortment of genomic segments of at least two different subtypes of influenza A viruses, which can result in an influenza pandemic. The 2009 influenza pandemic occurred when an A/H1N1 virus emerged from a triple-reassortant containing genes from avian, human, and swine influenza viruses (Michaelis et al., 2009). During this pandemic, several adjuvanted and unadjuvanted vaccines were developed. The adjuvanted vaccines contained a reduced amount of antigen per dose to increase the manufacturing capacities for the worldwide vaccine supply (Abelin et al., 2011). In addition to H1N1 vaccines, several candidate pre-pandemic H5N1 mock-up vaccines have been produced in the past with the aim of accelerating the regulatory processes for licensure of these vaccines in case of a H5N1 pandemic.

For vaccine formulation, release, and stability testing, the major antigenic envelope protein, HA, must be accurately quantified. The “gold standard” test used to determine HA in inactivated influenza vaccines is the Single-Radial ImmunoDiffusion (SRID) assay. This modified Ouchterlony technique is based on immunodiffusion of the antigen into an agarose gel containing a specific anti-serum (Schild et al., 1975; Wood et al., 1977). New HA reference antigens and anti-sera are prepared and calibrated by the Essential Regulatory Laboratories (ERL) each time a new virus strain is introduced into the vaccines. This calibration is described by the Word Health Organization (WHO, 2012). SRID has been used for influenza vaccine manufacturing for almost four decades. SRID has replaced less reliable tests, which were based on HA-induced aggregation of erythrocytes (Schild et al., 1975). Correlation has been demonstrated between SRID-measured vaccine potency and vaccine immunogenicity in clinical trials (Cate et al., 1983; Hobson et al., 1972; Wright et al., 1983).

While the SRID is the reference assay accepted by regulatory agencies to assess HA content and potency in influenza vaccines, the technique is very time consuming with at least 10 manual steps conducted over 2 days, from the preparation of the plates and agarose gel to the plate staining and ring readings (Fig. 1). In terms of sensitivity, the limit of quantification is typically evaluated to be approximately 5 µg/mL for unadjuvanted monovalent influenza vaccines. Furthermore, when HA concentration is measured in monovalent vaccines adjuvanted with aluminum salt-based adjuvants (i.e., AlOOH) by SRID, complex pre-treatments are required to desorb the HA antigen from the adjuvant (Sizer et al., 2014). SRID is also difficult to apply to emulsion-adjuvanted influenza vaccines due to interference with the diffusion of the antigen in the agarose gel. Therefore, alternatives to the standard SRID assay needed to better quantify HA in adjuvanted influenza pandemic vaccines, which may contain as little as 1.9 µg of HA/dose (Schubert, 2009).

In this context, we developed a simple, sensitive and rapid alternative assay, the Latex Agglutination Assay (LAA) to determine the concentration of HA in low-dose adjuvanted vaccines. This new analytical method can be used on a routine basis to quantify samples within two hours (Fig. 1).

## 2. Materials and methods

### 2.1. Monovalent influenza vaccines tested by LAA

Monovalent influenza vaccines corresponding to strains A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004, A/Vietnam/1194/04 (H5N1) NIBRG-14, A/Indonesia/05/05 (H5N1) NIBRG-2 or A/California/07/09 (H1N1) were prepared following the traditional embryonated egg production process in Sanofi Pasteur (Val de Reuil, France) from the reference master seed lots given to the vaccine manufacturers by the National Institute for Biological Standards and control (NIBSC, Potters Bar, UK) or the Center for Disease Control (CDC, Atlanta, US). Various adjuvanted influenza vaccines were also analyzed in this study: the A/California/07/09 (H1N1) adjuvanted with AF03, a squalene emulsion, the A/Vietnam/1194/04 (H5N1) NIBRG-14 and the A/Indonesia/05/05 (H5N1) NIBRG-2 adjuvanted with AlOOH or AF03.

### 2.2. Reference reagents used in LAA

SRID reference sheep antisera were used to coat the beads: A/New Caledonia/20/99 NIBSC code 04/260, A/Wisconsin/67/2005 NIBSC code 05/174, B/Malaysia/2506/04 NIBSC code 05/236, A/Vietnam/1194/04 (H5N1) NIBRG-14 NIBSC code 05/204, A/Indonesia/05/05 (H5N1) NIBRG-2 NIBSC code 03/212 or A/California/7/2009-like NIBSC code 09/194.

### 2.3. Principle of the LAA

The principle of the Spherotest® is described in Fig. 2. Briefly, when the anti-HA antibody-coated beads were mixed with a sample containing influenza vaccine, their aggregation with the HA causes a decrease of light absorption at an optical density (OD) of 405 nm. The OD is inversely proportional of the HA quantity.

### 2.4. Preparation of the beads

Optically active R9331 latex beads from Indicia Biotechnology (Saint Genis l'Argentière, France) with a diameter of 0.741 µm were used. Strain specific anti-sera were purified on a protein G column and purified IgGs were adsorbed passively onto the beads or linked covalently to them. Various amount of purified IgGs per gram of beads were assessed.

### 2.5. Zwittergent treatment

Influenza monovalent vaccine samples were treated with 0.01, 0.05 or 0.1% (W/V) of Zwittergent 3–14 (Calbiochem, Merck, Fontenay sous Bois, France). For this purpose 50 µL of the sample were mixed with 50 µL of 0.1, 0.5 or 1% (W/V) of Zwittergent 3–14 in 400 µL of the bead dilution buffer containing phosphate buffer pH 7.4, 0.1 mg/mL bovine serum albumin and 0.9% sodium azide. The mixture was then incubated 30 min at room temperature (RT).

### 2.6. Development of the LAA

A volume of 100 µL of the 10-fold diluted monovalent vaccines prepared previously was loaded in the first well of the first column of a 96-well plate. Two-fold serial dilutions were then performed in the bead dilution buffer. The reference (a monovalent vaccine of the same strain titrated in SRID), a positive control (another monovalent vaccine of the same strain titrated by SRID) and a negative control (beads with