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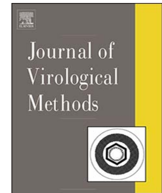

A latex agglutination assay to quantify the amount of hemagglutinin protein in adjuvanted low-dose influenza monovalent vaccines

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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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(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 antisera 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

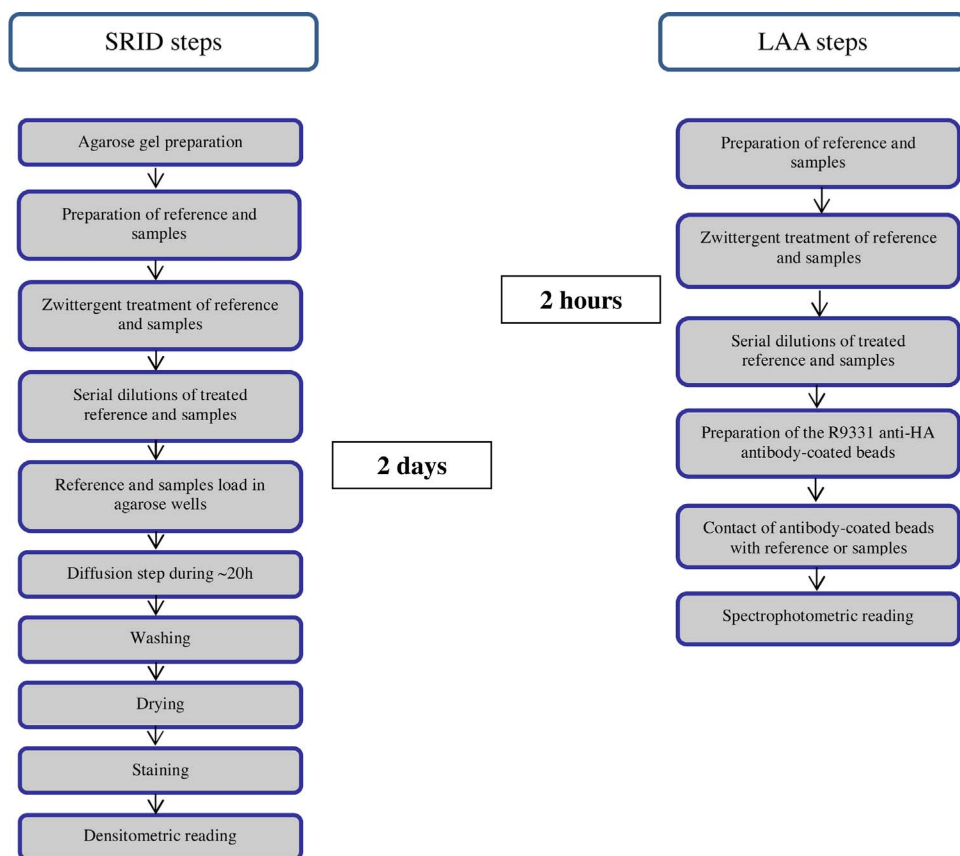

Fig. 1. Description of the various steps and approximate duration of the SRID and LAA techniques.

dilution buffer) were also 2-fold serially diluted and added to each plate.

Antibody-coated beads were extemporaneously diluted 1:4 with the bead dilution buffer and were homogenized by vigorous stirring for at least 60 s just before dispensing.

A volume of 20 µL of the diluted beads was then added into each wells of a new 96-well flat bottom microplate. Another 20 µL of each sample dilution previously prepared was then added to the beads. The plate was agitated on a microplate shaker for 5 min at RT at a speed of 1000 revolutions per minute (rpm). A volume of 100 µL of the stopping buffer (containing 15 mM NaCl and 0.1% tween) was added in each well and the plate was shaken for another 5 min at 600 rpm.

The optical density was immediately measured at 405 nm with a microplate spectrophotometer (e.g. Versamax, Molecular Devices, Sunnyvale, CA, USA).

The monovalent vaccine previously titered by SRID was used as a reference. The relationship between the reported optical density and the corresponding reciprocal standard dilution was modeled as a 4-parameter regression with Softmax pro (Molecular Devices, St Grégoire, France). The HA concentrations of the test samples, including the internal positive control, were calculated using the reference curve.

2.7. SRID

The SRID assay for determining the HA content of the different unadjuvanted monovalent samples was performed as described previously

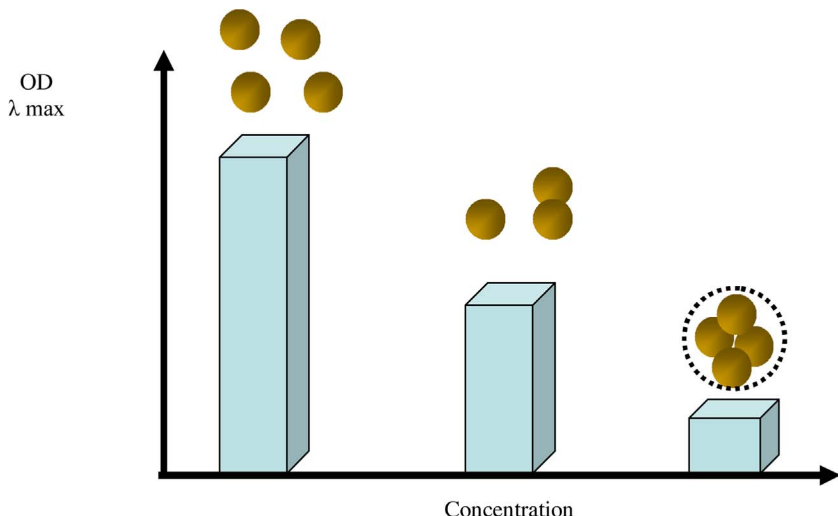

Fig. 2. Principle of the Spherotest®.
Single non-agglutinated submicron microspheres present a maximum of absorption at their OD max which depends on the refractive indices of both microspheres and environmental buffer, and on the diameter of the microspheres. Therefore, the decrease of the number of single microspheres can be optically monitored and quantified. In contact with the antigen, sensitized microspheres agglutinate to form clusters of bigger apparent diameter where they do not absorb at the same OD max.

(Legastelois et al., 2011) as adapted from Schild et al. (1975) and Wood et al. (1977). Briefly, the samples were first incubated with 1% (w/v) Zwittergent 3–14 for 30 min at room temperature (RT) and subjected to immunodiffusion for approximately 20 h (h) at RT in an antibody-loaded agarose gel. The diameters of the precipitation rings of antigen–antibody complexes were measured utilizing an Epson Professional scanner 1680 (Microvision, Evry, France). The final titers were calculated from the calibration curve of a reference whole virus batch preparation of known HA concentration obtained from the NIBSC, using the Immulab 4.2 software. Each sample was titrated at least three times on three dilutions. Under these conditions, the precision of the SRID is estimated to be $x/1.2$ with a range of quantification of ~5–30 µg HA/mL.

2.8. Assessment of the analytical performance parameters

2.8.1. Linearity

Three separate series were performed by two operators on different days. Each run included the assay of a range of 5 or 6 concentrations of HA respectively for the AF03-adjuvanted A/California/07/09 (H1N1) vaccine and the unadjuvanted and AF03-adjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines. For each vaccine, the SRID titers were known and independent dilutions were performed. The theoretical expected concentration was calculated for each dilution level.

The linearity over the chosen range was tested through the following steps: i) the homogeneity of bound variances was verified by COCHRAN's test, ii) the dependence between the theoretical expected concentration measured and the linearity of this relation was tested using an unweighted linear regression using least squares method. A significant slope ($p\text{-value} < 0.01$) and non-significant deviation from linearity must be demonstrated ($p\text{-value} > 0.05$).

The limit of quantification was defined as the lower concentration of the linearity domain.

2.8.2. Accuracy

Accuracy was obtained from the equation: % of recovery = (observed concentration/theoretical expected concentration)*100, i.e. by calculating all recovery percentages obtained between the observed concentrations measured in LAA and the theoretical expected concentrations related to SRID from the same samples and the same experimental design used in the linearity study.

The method was considered accurate if all recovery percentages are included within the equivalence limits [80%; 120%].

2.8.3. Precision

The precision is evaluated through repeatability (intra-assay) and intermediate precision (inter-assay). Three independent runs in intermediate precision conditions, each including three independent measurements in repeatability conditions were performed to assess both parameters. For the A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines, various concentrations were tested: 60 µg HA/mL for the AlOOH-adjuvanted vaccines and 3.8, 15 and 50.9 µg HA/mL for the unadjuvanted and AF03-adjuvanted vaccines. For the AF03-adjuvanted A/California/07/09 (H1N1) vaccine, both 7.6 and 15 µg HA/mL concentrations were tested. The precision was expressed as the 95% confidence interval of intermediate precision for one run with one measurement.

2.8.4. Inter-laboratory reproducibility

A comparison between the results of two independent laboratories A and B (two different operators, two different lab facilities) was performed with the A/Indonesia/05/05 (H5N1) NIBRG-2 strain unadjuvanted or adjuvanted with AF03 or AlOOH. The three samples were titrated in two separate series of six replicates. The accuracy is determined by the percentage of recovery calculated between the means of the HA concentrations determined for the two independent laboratories. The F-test and t-test were performed respectively on the variance and the mean of the concentrations.

2.8.5. Statistical analyses

All the statistical processing was performed under SAS v9.1 software (SAS Institute Inc, USA).

In order to obtain a normal distribution of the data, a logarithmic transformation was applied to the titers.

3. Results

3.1. Development of the LAA

The development of the LAA was mainly performed following the supplier recommendation with some optimizations. First, two types of binding on beads of the IgGs purified from the reference NIBSC sera were evaluated, a passive adsorption and a covalent adsorption. For each type of binding, two amounts of antibodies were tested: 20 and 40 mg per gram of beads. This optimization was carried out using beads-coated with three reference epidemic strain serums: A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. The choice of the type of beads was based on the best agglutination percentage between beads and antigen traduced by the highest OD difference between the negative and the positive control.

The best result was obtained with 40 mg of purified anti-serum per gram of beads, passively adsorbed (data not shown). Moreover, the volume of the reaction was also optimized: 20, 50, 100 µL of beads and 20, 50 100 µL of Ag were assessed, as the lower the reaction volume, the shorter the incubation time will be. Finally 20 µL of beads and 20 µL of antigen were chosen.

Various concentrations of Zwittergent 3–14 0.1, 0.05 and 0.01% were then tested using the A/Indonesia/05/05 (H5N1) NIBRG-2 monovalent vaccine samples adjuvanted or not with AlOOH (Table 1). One percent Zwittergent 3–14 is currently used in the SRID assay to disrupt the virions and solubilize the viral HA proteins anchored in the lipid envelope to allow the quantification of the HA. It is also used to separate viral proteins from the AlOOH adjuvant (Sizer et al., 2014). The optimal concentration of Zwittergent used to obtain highly reproducible LAA titers and accurate quantification of AlOOH adjuvanted monovalent vaccine was 0.01%. Higher concentrations of detergent (0.1 and 0.05%) resulted in complete inhibition of agglutination. The reference monovalent and the positive control corresponding to each influenza strain were also treated with 0.01% Zwittergent before quantification.

A LAA representative graph for the unadjuvanted A/Vietnam/1194/044(H5N1) NIBRG-14 monovalent vaccine, after LAA optimization, is presented in Fig. 3.

Table 1
HA quantification by LAA in A/Indonesia/05/05 (H5N1) NIBRG-2 vaccine samples adjuvanted or not with AlOOH using various% of Zwittergent 3–14.

% Zwittergent 3–14	Bead agglutination with antigen	LAA titer µg/mL H5N1 vaccine unadjuvanted (143 µg/mL in SRID)	LAA titer µg/mL H5N1 vaccine AlOOH (70 µg/mL in SRID)
0.1	–	*	*
0.05	–	*	*
0.01	+ + +	**165**	**72**
0	+ + +	123	16

The SRID concentrations are given for each vaccine in brackets.
An estimation of the agglutination of the beads is also shown.
[–]: No bead agglutination.
[+ + +]: Bead agglutination.
[*]: No titer
Bold letters: optimal concentration of Zwittergent.

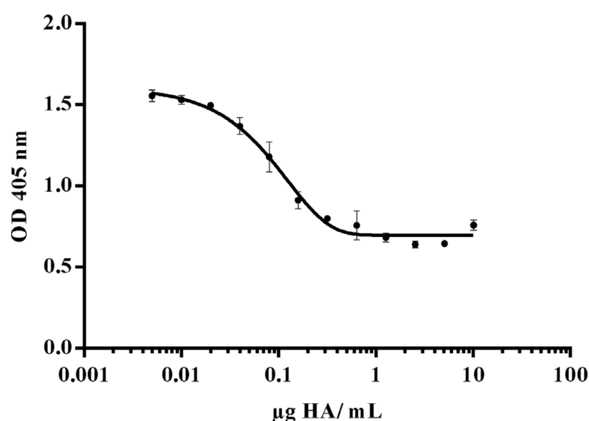

Fig. 3. Graph of OD values at 405 nm against HA concentrations for the unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 monovalent vaccine after optimization of the LAA technique.

Vaccine was at 1/10 dilution in the first well followed by serial twofold dilutions. Data are representative of at least three independent experiments. Each point in this curve is a mean ± standard error of mean.

3.2. HA quantification in different influenza monovalent vaccines

After the development of the LAA, the HA quantification in several influenza monovalent vaccines was estimated by this method and compared to the reference assay, SRID. As shown in Table 2, the LAA titers obtained were very close to the SRID titers with a ratio (SRID titer/LAA titer) between 0.87 and 0.99. The limit of detection was also assessed for various monovalent vaccines. As expected for such assays, the limit of detection was very low and could detect as little as 15 ng HA/mL (data not shown).

These results showed that the LAA could be considered as an alternative technique to SRID for HA quantitation and the performance parameters were next evaluated.

3.3. Assessment of the analytical performance parameters

3.3.1. Linearity of LAA

LAA was linear on the tested dose ranges between 1.8–50.9 µg HA/mL for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines (Fig. 4). LAA was linear between 3.4–43.8 µg HA/mL for the AF03-adjuvanted A/California/07/09 (H1N1) vaccine (data not shown). All acceptance criteria were satisfied.

The limit of quantification was defined as the lowest concentration of the linearity domain: 1.8 µg HA/mL for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines and 3.4 µg HA/mL for the AF03-adjuvanted A/California/07/09 (H1N1) vaccine. These results confirmed that the quantification limit of the LAA method is lower than the SRID method, which in our hands is approximately 5 µg. HA concentrations below 1.8 µg HA/mL were intentionally not tested since influenza vaccines do not contain less than 1.8 µg HA/mL.

Table 2
HA quantification by LAA in various monovalent vaccines.

Monovalent vaccine	SRID titer (µg/mL)	LAA titer (µg/mL)	Ratio SRID titer/ LAA titer
A/NC/20/99 (H1N1)	119.9	130.5	0.92
A/Wisconsin/67/05 (H3N2)	141.0	158.4	0.89
B/Malaysia/2506/04	130.0	131.5	0.99
A/California/07/09 (H1N1)	15.0	16.9	0.89
A/Vietnam/1194/04 (H5N1) NIBRG-14	101.8	102.8	0.99
A/Indonesia/05/05 (H5N1) NIBRG-2	143.0	165.0	0.87

A ratio between the titers of the two assays is mentioned.

3.3.2. Accuracy of LAA

For each concentrations tested, the recovery percentage between the obtained concentrations in LAA and the respective expected concentrations determined by SRID was between 80 and 120%. It was concluded that the LAA was accurate from 1.8 to 50.9 µg HA/mL for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines (Fig. 5). Accuracy of the LAA was also confirmed for the AF03-adjuvanted A/California/07/09 (H1N1) vaccine with a global mean percent recovery of 91%.

3.3.3. Precision of LAA

For both pandemic vaccines, A/California/07/09 (H1N1) and A/Vietnam/1194/04 (H5N1), the intermediate precision of the LAA evaluated for one run and one measurement was lower than the SRID assay precision: $x/1.2$ for the SRID, more than $x/1.3$ for the LAA. However, when three independent experiments were performed, the LAA precision was similar to the SRID (Table 3).

3.3.4. Inter-laboratory reproducibility

The reproducibility was performed on another pandemic vaccine, namely the A/Indonesia/05/05 (H5N1) vaccine previously tested unadjuvanted in Tables 1 and 2. For each A/Indonesia/05/05 (H5N1) vaccine (unadjuvanted, AlOOH or AF03-adjuvanted), the percentages of recovery calculated between the means of the 12 HA concentrations determined by two independent laboratories were all included between 80% and 120% (Table 4). The *F*-test and *t*-test showed no significant difference between the two laboratories concerning respectively, the variance and the mean of the concentrations. These data indicate that the LAA is reproducible with similar results obtained in different laboratories.

4. Discussion

The reference SRID assay used to determine HA content in influenza vaccines is labor intensive and time consuming. The present study was conducted to develop a simple, sensitive and rapid alternative assay method that could be used on a routine basis to quantify influenza vaccine samples within two hours. Unlike other latex bead agglutination assays previously described for detecting influenza viruses (Chen et al., 2007), the LAA presented here is based on the Spherotest® technology, which produced accurate HA quantification of seasonal and pandemic monovalent vaccines.

The qualification study performed demonstrated that the LAA is suitable to quantify HA as the LAA method is linear, accurate when compared to SRID. The precision is close to that of the SRID assay with a limit of quantification lower than that of SRID. Furthermore, the LAA was found to be reproducible from one laboratory to another. Moreover, LAA appears to quantify HA in its native conformation. To demonstrate this, complementary studies were performed using monovalent influenza vaccine samples submitted to heat degradation showing a decrease of HA titers measured by LAA (data not shown). These results are of interest since change of HA conformation has been shown to result in a loss of immunogenicity (Minor, 2015; Williams, 1993) and cannot be assessed with other methods such as HPLC and mass spectrometry.

During the LAA development, its specificity was also assessed. It was observed that the IgGs purified from the polyclonal reference sera used to coat the beads could recognize other strains probably due to the sensitivity of the LAA (data not shown). Hence, the method was mainly developed for monovalent pandemic vaccines and cannot be used to quantify each monovalent in a trivalent or quadrivalent vaccine.

One particular feature of the LAA is the determination of the amount of HA in low-dose adjuvanted influenza vaccines. The AlOOH or AF03 adjuvants have been used to formulate A/H5N1 influenza vaccines (Bresson et al., 2006; Levie et al., 2008). Given that A/H5N1 vaccines were shown to not be as immunogenic in humans as seasonal A

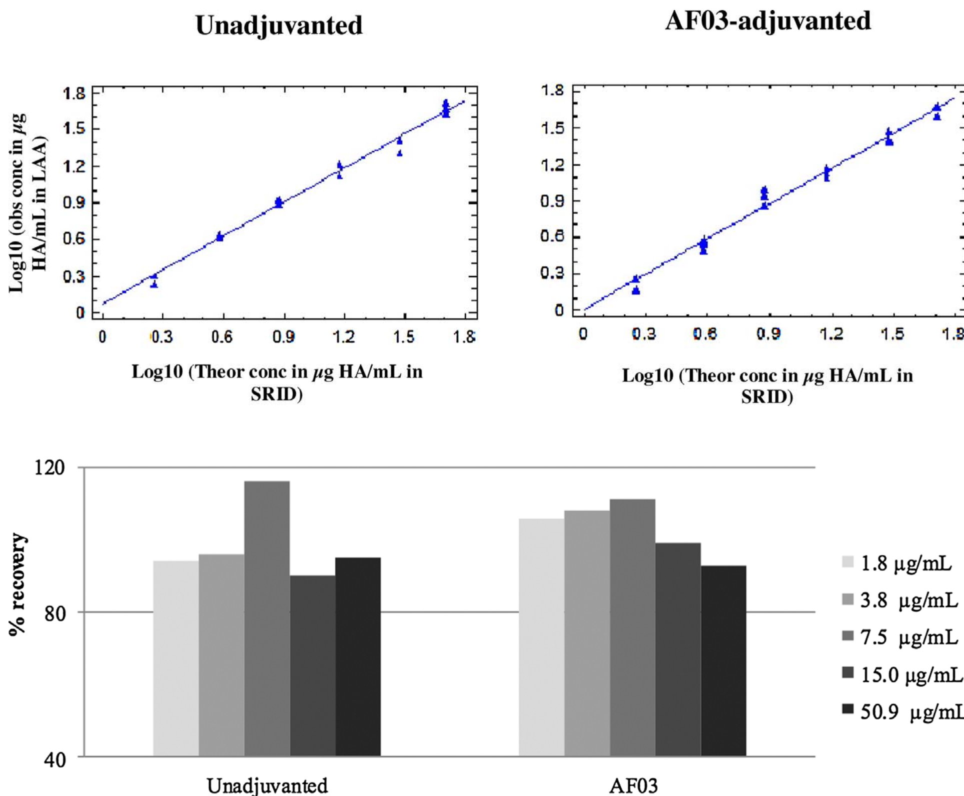

Fig. 4. Linearity between the observed concentrations obtained in LAA compared to the theoretical expected concentrations obtained in SRID for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines.
The linearity was tested on the dose ranges between 1.8–50.9 μg HA/mL for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBRG-14 vaccines.

Fig. 5. Accuracy of the LAA for the AF03-adjuvanted and unadjuvanted A/Vietnam/1194/04 (H5N1) NIBG14 vaccines: Recovery percentages compared to the SRID.
The method was considered accurate if all recovery percentages are included within the equivalence limits [80%; 120%].

Table 3
Precision of the LAA.

Monovalent vaccine	Adjuvant	Intermediate Precision (fold) For 1 measurement	Repeatability (fold) For 3 independent measurements
A/California/07/09 (H1N1)	AF03	0.11 (x/1.30)	ND[a]
A/Vietnam/1194/04 (H5N1) NIBRG-14	No adjuvant or AF03	0.15 (x/1.41)	0.09 (x/1.22)
	AlOOH	0.14 (x/1.38)	0.08 (x/1.2)

The precision was evaluated through intermediate precision (inter-assay) and repeatability (intra-assay). Three independent runs in intermediate precision conditions, each including three independent measurements in repeatability conditions were performed to assess both parameters.
[a] Not determined.

Table 4
Inter-laboratory reproducibility of the LAA.

Strain	Adjuvant	Percentage of Recovery
A/Indonesia/05/05 (H5N1) NIBRG-2	No adjuvant	96%
	AlOOH	101%
	AF03	98%

Accuracy was determined by the percentage of recovery calculated between the means of the HA concentrations determined for two independent laboratories. The three samples were titrated in two separate series of six replicates.

influenza strain vaccines, probably due to the absence of subtype-specific immune priming, influenza vaccine producers have primarily focused on the preparation of adjuvanted vaccines to increase vaccine immunogenicity, to induce cross-strain reactive antibodies and to reduce the vaccine dose (Keitel and Atmar, 2009). The same approach was taken during the 2009 A/H1N1 pandemic, except that this pandemic strain appeared to be as immunogenic as the seasonal A influenza strains. Hence the role of the adjuvant was essentially for antigen dose sparing in this case (Schubert, 2009). Our results suggest that the LAA could be used to quantify the amount of HA in vaccine batches adjuvanted with AlOOH or AF03 as efficiently as in unadjuvanted vaccine.

For emulsion-based adjuvanted vaccines, it has been shown that a dose of 1.9 μg of HA (3.8 μg/mL) is sufficient to induce a protective immunogenicity even though the trivalent or quadrivalent epidemic vaccines are formulated at 15 μg of HA of each strain per dose without adjuvant (SAGE Working Group, 2009; Vogel et al., 2009). SRID, with quantification limit around 5 μg HA/mL, is not an ideal assay for the determination of HA in the low-dose pandemic vaccines. In contrast, LAA can accurately measure low doses of HA contained in these vaccines, since the limit of quantification of the LAA was determined at around 2–3 μg of HA/mL (not tested below) and the sensitivity around 15 ng of HA/mL for the adjuvanted and unadjuvanted monovalent vaccines.

The SRID assay similarly to the LAA described here, relies on the availability of reference materials *i.e.* the HA antigen reference and corresponding anti-serum. These reagents are updated and distributed annually by the ERL. It takes in general 2–3 months to prepare and calibrate them. During a pandemic influenza situation, the lack of availability of the reference materials would slow down vaccine development. Other antibodies than the purified IgGs from the reference anti-serum could be coated on the latex beads as universal antibodies, e.g., IgM monoclonal antibodies which recognize all the HA subtypes in egg-derived vaccines (Legastelois et al., 2011), cross-reactive antibodies directed to the HA stalk region (Ekiert et al., 2011; Hufton et al., 2014), universal antibodies recognizing non-conformational epitopes (Chun et al., 2008) or fetuin/lectins mimicking influenza receptors (Hashem et al., 2013; Mandenius et al., 2008).

A number of alternative assays to SRID have been suggested. High-performance liquid chromatography (HPLC) (Lorbetskie et al., 2011) and mass spectrometry (Creskey et al., 2012; Getie-Kebtie et al., 2013) have been proposed for HA quantification.

Surface plasmon resonance (SPR) has also been developed to

quantify HA in the influenza vaccines. Some investigators have designed SPR assays using lectins as sensor ligands but such assays are not strain specific (Jiang and Eichelberger, 2015; Mandenius et al., 2008). Another group has developed a method of HA quantitation *via* an inhibition assay using HA proteins for H1N1, H3N2 and B strains immobilized on a sensor chip (Nilsson et al., 2010). All these methods require complex and expensive instrumentation and highly trained personnel, which is not the case for the LAA.

Another antibody-based method has been designed, the VaxArray™. The VaxArray™ Influenza potency assay is a multiplexed immunoassay for rapidly quantifying HA in seasonal influenza vaccines for potency determination. The assay is based on a "universal" panel of subtype-specific monoclonal antibodies printed in an array format. However, the assay requires a specific microarray reader, associated lab ware and a calibration of the reference (Kuck et al., 2017).

Various Enzyme-Linked ImmunoSorbent Assays (ELISA) for measuring vaccine HA concentration have been proposed (Bodle et al., 2013; Chun et al., 2008; Hashem et al., 2013; Legastelois et al., 2011). In theory, all these ELISA-based methods described in the literature could potentially be adapted to the LAA. An advantage of the LAA, compared to classical ELISA, is that the recognition between the HA in the vaccine and the beads occurs in suspension, allowing a better interaction between antigen and anti-serum.

Today all these alternatives technologies especially mass spectrometry and SPR are presented as potential candidates to replace SRID, with collaborative studies conducted by several vaccine manufacturers and ERLs through potency assay working groups (NIBSC, 2017).

We believe that LAA may be useful to rapidly and accurately quantify the influenza HA protein in monovalent vaccines especially in those containing less than 5 μg/mL of HA in the presence of an adjuvant. Application of the LAA for the quantification of recombinant HA proteins or Virus like particles produced in various expression systems, are currently being evaluated. Development of this assay to determine HA production yields as an industrial process monitoring test is envisaged as well.

Declaration of interest

Sophie Buffin, Nabila Ikhelef, Joseline Dubayle, Nolwenn Nougarede, Catherine Moste, and Isabelle Legastelois are employees of Sanofi Pasteur. Marie-Pierre Varenne is an employee of Indicia Biotechnology and Julien Prudent is an employee of the University of Cambridge.

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