Original Article

Optimization and Validation of ELISA for Pre-Clinical Trials of Influenza Vaccine

(ELISA / influenza / validation / pandemic)

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Abstract. Testing of every new vaccine involves investigation of its immunogenicity, which is based on monitoring its ability to induce specific antibodies in animals. The fastest and most sensitive method used for this purpose is enzyme-linked immunosorbent assay (ELISA). However, commercial ELISA kits with whole influenza virus antigens are not available on the market, and it is therefore essential to establish an adequate assay for testing influenza virus-specific antibodies. We developed ELISA with whole influenza virus strains for the season 2011/2012 as antigens and validated it by checking its specificity, accuracy, linearity, range, precision, and sensitivity. The results show that we developed high-quality ELISA that can be used to test immunogenicity of newly produced seasonal or pandemic vaccines in mice. The pre-existence of validated ELISA enables shortening the time from the process of vaccine production to its use in patients, which is particularly important in the case of a pandemic.

Introduction

Every year, influenza virus types A and B cause human outbreaks that are responsible for substantial mortality and morbidity, particularly in high-risk groups, such as the elderly, infants, and immunocompromised individuals. Prevention is the most effective method of reducing transmission and the socio-economic burden of influenza (Szucs et al., 2001). Protection against influenza virus is primarily mediated by antibodies directed against the influenza haemagglutinin (HA) and neuraminidase (NA) (Gillim-Ross and Subbarao, 2006; Asconas et al., 1982). HA is responsible for attachment and penetration of the viral particles into cells during the initial stages of infection. On the other hand, NA is associated with the release of nascent virions from the cell membranes (Schulman and Palese, 1977). Annual influenza epidemics and pandemics represent the result of antigenic changes in HA and NA molecules, known as antigenic drift and shift. During the drift, HA and NA surface antigens undergo progressive amino acid substitutions that can result in evasion of the previously acquired immunity (Mann, 2006). An additional complication to combating influenza infection is the increase in measured resistance to the standard antiviral drugs, amantadine and rimantadine (Bright et al., 2005, 2006). Therefore, influenza vaccine is still the best prevention against influenza.

Since 1977, influenza A (H3N2) and (H1N1) subtypes have co-circulated with influenza B viruses in human population. For each season, World Health Organization (WHO) prepares instructions for vaccine manufactures about new vaccine formulations to match the most prominent circulating strains.

Virions for influenza vaccines are usually produced in hen eggs or in cell culture. After virus inactivation, whole or split virions are used for vaccine production. Recent progress in molecular biology techniques has introduced changes in vaccine manufacturing such as using recombinant biotechnology for virus antigen production (Sedova et al., 2012). Regardless of the virion production technology used, according to the European Medicines Agency (EMA) and national authority Medicines and Medical Devices Agency of Serbia (ALIMS), each newly produced vaccine must be tested in preclinical studies for immunogenicity before the use in clinical trials. However, both production and checking processes are time-consuming. Thus, it is important to have al-

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Abbreviations: ALIMS – Medicines and Medical Devices Agency of Serbia, BSA – bovine serum albumin, CV – coefficient of variation, ELISA – enzyme-linked immunosorbent assay, EMA – European Medicines Agency, HA – haemagglutinin, HI – haemagglutination inhibition, MN – microneutralization, NA – neuraminidase, PBS – phosphate-buffered saline, SD – standard deviation, TTd – tetanus toxoid, WHO – World Health Organization.

ready prepared and standardized tests for detection of antibodies produced in experimental animals after immunization with a newly manufactured vaccine. This shortens the time required for approval of vaccines by national authorities and the following immunization of humans, which is particularly important in the case of a pandemic.

Haemagglutination inhibition (HI) and microneutralization (MN) assays are typically used for antibody titre estimation. These methods are used to measure the functionality of induced antibodies (Abs), but they are complicated to perform (especially MN). On the other hand, enzyme-linked immunosorbent assay (ELISA), although it cannot serve as a measure of antibody functionality, can detect the presence of specific antibodies in significantly higher sera dilutions compared to HI and MN. This makes it a more sensitive method (Turner et al., 1982; Yoon et al., 2004, Schmitz et al., 2013), which is at the same time easy to perform. Moreover, ELISA allows testing of individual isotypes of antibodies specific for the influenza virus, and interpretation of the results rests on absorbance readings, instead of subjective visual estimations of agglutination (Alvarez et al., 2010). In that way, it can be said that ELISA, HI and MN make a set of methods for detection and quantification of anti-influenza antibodies (Descoteaux et al., 1980; Turner et al., 1982).

When an ELISA kit to a particular virion influenza antigen does not exist, in circumstances of time constrains, brought about by influenza pandemics for example, there is a lack of possibility to develop such a kit for animal sera, validate it and make it commercially available fast enough. Therefore, it is useful to have a validated ELISA to the whole influenza virus, because the state of the antigen used for immunization (whole or split virus) in both cases generates antibodies that recognize whole-virus epitopes. That is why we have developed and validated ELISA with the whole-virion antigens that can be used to detect both influenza virus-specific mouse IgG antibodies and influenza virus-specific IgG antibody isotypes: IgG1, IgG2A, IgG2B or IgG3.

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Typical validation characteristics that should be considered are specificity, accuracy, linearity, range, precision, and sensitivity. The validation of our ELISA was performed according to the guidelines of EMA (ICH M5 EWG, 2005) and done by determination of the abovementioned characteristics. The values of all parameters showed that our method is characterized by very high quality. Our ELISA enables quick and easy application in monitoring the immune response in mice after immunization with a newly developed influenza vaccine.

Material and Methods

Animals

Ten-week-old Swiss white female mice were used in the experiments. We formed two groups of animals (10 mice per group). One group of mice was immunized with trivalent split vaccine against influenza (Sanofi Pasteur S. A., Lyon, France) (10 μg HA per dose), and the other group received the same volume of phosphate-buffered saline (PBS). The vaccine was produced for the season 2011/2012 for the northern hemisphere according to WHO recommendations. The volume of 100 μl of the vaccine or PBS per mouse was administered once intramuscularly, 50 μl in each *musculus quadriceps*. In order to prepare the control serum for determination of the assay specificity, the mice of the same strain and age were immunized with an independent antigen, tetanus toxoid (TTd), 100 μg/dose, 200 μl/mouse, subcutaneously.

Mice were raised under conventional conditions. All animal experimentation was conducted in accordance with Serbian Law of animal welfare, published in Official Gazette RS (Službeni glasnik RS, No. 41/2009 of June 2, 2009). Our experimentation received the approval No. 323-06-03742/2012-05 of the Animal Institutional Care and Use Committee at the Institute of Immunology and Virology "Torlak", based on permission of the Ministry of Agriculture, Forestry and Water Management, Veterinary Administration of the Department of Animal Welfare.

Samples of blood were collected four weeks after immunization by bleeding from the retro-orbital plexus under ketamine/xylazine anaesthesia. The collected sera were complement depleted, aliquoted and stored at –20 °C until used for analyses. Thereafter, we made the sera pool by mixing equal volumes of sera from immunized mice and marked as the standard.

ELISA

Whole inactivated viral antigens were obtained from the Influenza Vaccine Department of Institute of Virology, Vaccine and Sera "Torlak". Antigens were adsorbed to flat-bottom 96-well microtiter plates (Nunc MaxiSorp™ ELISA plates, Nunc, Roskilde, Denmark) for 18 h at 4 °C. The optimal concentration of each antigen in PBS (A H1N1, A H3N2, or B whole virion) was determined separately during the optimization process. After the adsorption step, the plate was blocked using 200 μl/well 2% bovine serum albumin (BSA) (SERVA Electrophoresis GmbH, Heidelberg, Germany) in PBS for 2 h at room temperature (RT). After washing three times with 0.05% Tween 20/PBS (Sigma-Aldrich Co., St. Louis, MO) and once with PBS (200 μl per well), standard sera in desired dilutions were added in duplicates.

In order to determine the optimal concentration of antigen and the level of accuracy, standard serum was diluted 1 : 120. In all tests, it was always diluted in 1% BSA/0.05% Tween 20/PBS, and 50 μl of serum dilution was applied per well.

The specificity of the assay was checked with sera diluted 1 : 500. To determine linearity we used serial twofold sera dilution starting from 1 : 32. The standard antiserum was used for optimization and in all valida-

tion steps. Sera from TTd-immunized mice were used for specificity determination. After 1 h of incubation at RT, the excess serum was washed from each well in the same manner as previously. Ag-specific serum IgG binding was detected by peroxidase-labelled goat anti-mouse IgG immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., Baltimore, PA) and the plates were incubated at RT for 1 h. After a subsequent rinse cycle to remove excess conjugate, substrate *o*-phenylenediamine dihydrochloride (OPD tablets; Sigma-Aldrich Co.) was added. After 15 min of incubation at RT, the absorbance (492/620 nm) of each well was determined by an automated reader (Labsystems Multiskan Ascent 384 Microplate Reader, MTX Lab Systems, Santa Clara, CA). All materials were used according to manufacturers' instructions.

Inhibitory ELISA

Inhibitory ELISA (Crowther, 2001) was performed as described above, with the exception of the serum addition step. The serial dilutions of antigen and standard serum were mixed in an equal volume to obtain final antigen concentrations starting from 8 to 0.5 μg/ml, and final serum dilution of 1 : 32. This mixture was incubated for 30 min at RT and added to microtiter wells. All further steps were performed as described previously.

Results

Optimization

First, we determined the optimal concentration of whole-virion antigens for binding to the surface of the microtiter plate (Fig. 1). The optimal concentration refers to one that provides the minimum possible amount of antigen that completely covers the bottom of the well of the microtiter plate. Further increase of antigen concentration does not lead to signal amplification (absorbance), but could form layers of antigens that may be unstable, leading to erroneous measurements. We determined the concentration of 2.5 μg/ml of HA as optimal for all three viral antigens, and this concentration of antigen was used in all further tests.

In addition to the concentration of antigen, the process of ELISA optimization includes determining other conditions for performing the test. These conditions are mostly standard, and rarely change when protein antigens are used. We tested optimal incubation time with blocking solution and with sera dilution (30 min to 2 h), optimal incubation temperature for blocking solution and for sera dilutions (RT or 37 °C), washing buffer composition (PBS or carbonate buffer, with or without Tween 20) (results not shown), sera dilution buffer composition (1% BSA/PBS or 1% BSA/% Tween/PBS), and different types of saturation agents (1% BSA, 2% BSA, or 10% normal horse sera) (Fig. 2) and we confirmed that the standard procedure outlined in materials and methods was best suited for the test performance.

Validation

Specificity

Specificity was determined in two ways: 1) by parallel testing of mouse sera obtained after immunization with influenza vaccine and obtained after immunization with independent antigen (TTd), and 2) by inhibition of standard antiserum binding to influenza virus in the presence of whole-virion antigens**.**

There was a significant difference between the measured absorbance in the serum of animals that were immunized with the influenza vaccine and those injected with TTd or PBS. Absorbance values obtained in sera of

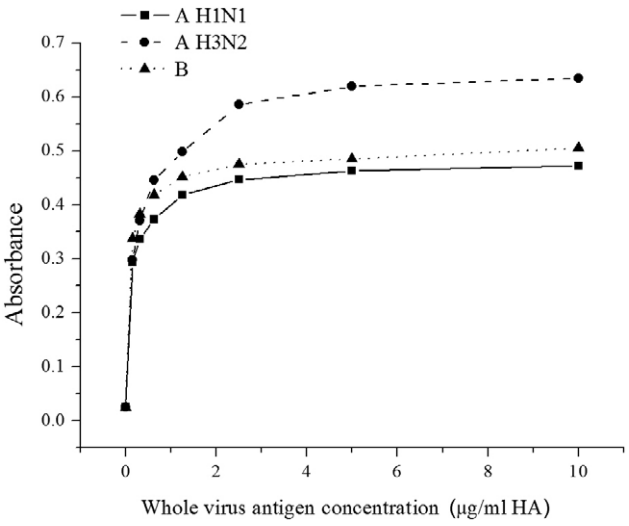

Fig. 1. Determination of influenza viral antigen (A H1N1, A H3N2 and B strains) optimal concentration for the use in whole-virion ELISA. All samples were assayed in duplicates and mean absorbances at 492/620 nm are presented.

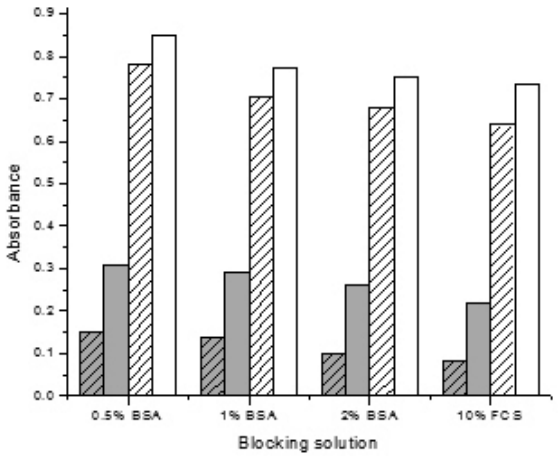

Fig. 2. Determination of optimal blocking solution and sera dilution buffer composition. Grey column with stripes: negative control sera in 1% BSA/0.05% Tween 20/PBS; grey column: negative control sera in 1% BSA/PBS; white column with stripes: standard sera in 1% BSA/0.05% Tween 20/PBS; white column: standard sera in 1% BSA/PBS.

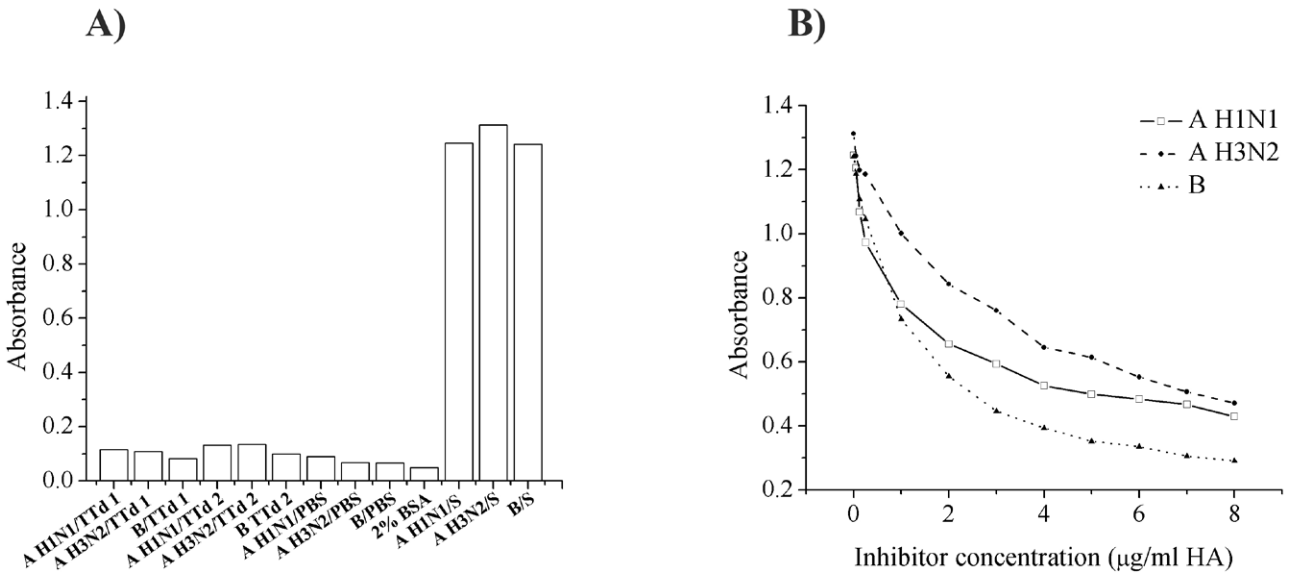

Fig. 3. Validation of specificity. **A**) Comparison of IgG antibodies for different influenza antigens (before slash) in standard mouse antisera (S), and sera taken from mice injected with TTd (TTd1, TTd2) or PBS (PBS) (after slash); 2% BSA – negative control. **B**) Inhibition of standard antiserum binding to viral antigens bound to the microtiter plate in the presence of increasing concentrations of specific viral antigens (inhibitors – A H1N1, A H3N2, or B). All samples were assayed in duplicates and mean absorbances (492/620 nm) are presented.

TTd- and PBS-injected mice were very close to the absorbance of the negative control (2% BSA was loaded into the well instead of serum), which confirmed the specificity of the assay (Fig 3A).

Additionally, a more direct and reliable way to test the specificity is to perform inhibitory ELISA (Fig. 3B). The appropriate serum dilution (1 : 32) used in this type of ELISA was determined from the curves representing the binding of serial twofold serum dilutions to corresponding virus antigens (Fig. 4A). This specific serum dilution was selected as optimal because it provided sufficiently high absorbance, but still did not reach the plateau of the curve. Before applying to the plate, the diluted serum was mixed with increasing concentrations of the specific antigen. In that way, the virus antigen bound to specific serum antibodies, thus reducing the amount of free antibodies of the same specificity remaining that could bind to the antigen on the plate. This resulted in reduction of the recorded absorbance with increasing concentration of antigen (inhibitor) used in the solution. The absorbance obtained in this way displayed an inhibitory curve, which indicated the presence

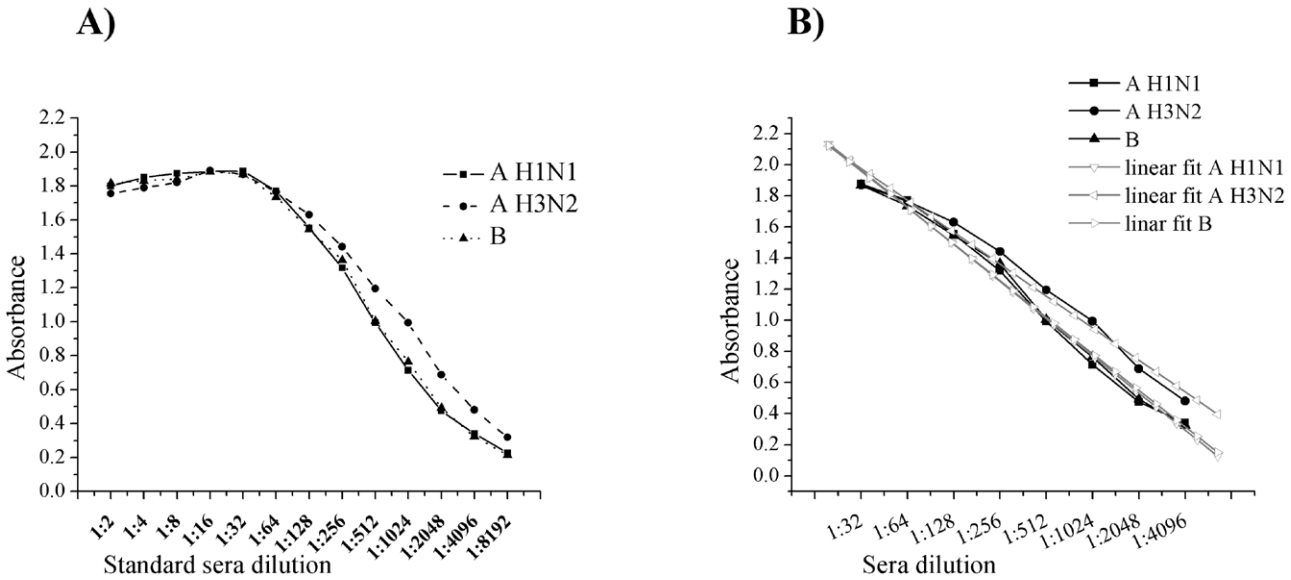

Fig. 4. Determination of linearity of ELISA for detection of antibodies specific for whole-virion antigens. **A**) IgG antibodies specific for A H1N1, A H3N2 and B strains of influenza virus in serial dilutions of the standard antiserum; **B**) determination of parameters of the linear regression equation for the range of linearity.

of specific antibodies in the tested serum (Fig. 3B). The reduction in absorbance in our inhibitory curve was shown for all three antigens.

Linearity, range and sensitivity

ELISA is also characterized by its **linearity**, which refers to the range of serum dilutions that provide the linear segment of the curve. This linear part of the binding curve can be used to quantitate the obtained absorbance. This characteristic was determined by using standard antiserum in twofold serial dilutions and its binding to the corresponding antigens (Fig. 4 and Table 1). The absorbance obtained for sera dilutions from 1 : 32 to 1 : 4096 (Fig. 4B) gave the linear part of the curve and was used to calculate parameters *a* and *b* from linearity equation y = *a* + *b* * x of the linear curve (Table 1). The coefficient of determination (R^2), which is used to describe the regression line of experimental data, was very high for all three antigens/viruses, about 0.98, i.e. overlap of the measured values with the linear curve was 98 % in this range (Table 1). In other words, the tested serum dilution that gives the absorbance in the range of that obtained for the standard antiserum dilutions from 1 : 32 to 1 : 4096 can be objectively measured. Measuring the absorbance outside this range therefore is not valid.

Linearity is used to determine the **range** of ELISA, which is defined as part of the curve in which there are enough acceptable values of linearity, accuracy, and precision. The range of the assay is limited by its upper and lower values. The upper limit is defined as the mean absorbance of 10 duplicate samples that reached the maximum in the linear part of the curve, subtracted by three standard deviations. By analogy, the lower limit is defined as the mean absorbance of the samples that reached the minimum in the linear part of the curve, with addition of three standard deviations. The values of absorbance of the upper and lower limits of our ELISA are shown in Table 2. This absorbance was recorded for the dilutions of standard antiserum of 1 : 64 for the upper limit, and 1 : 4096 for the lower limit, which was the case for all three tested viral antigens.

Sensitivity (limit of detection) is defined as the smallest amount of the analysed substance which can be distinguished from the absence of that substance (a *blank value*) by the method under evaluation. Sensitivity of other analytical procedures, even other ELISAs (sandwich ELISA), is defined as the least amount of analyte that can be detected. The sensitivity is then calculated from the standard deviation of the blank (negative control) and the linear slope of the curve. If L_D is the label for the limit of detection, SD(blank) label for standard deviation of the blank, and b is the slope of the linear curve (y= a + bx), then $L_D = 3.3(SD(blank)/b)$ (European Medicines Agency, EMA, ICH EWG, 2005). According to this calculation, the limit of detection of our ELISA was a little lower than the lower linearity limit (Fig. 4 and Table 2). The nature of the Ag/Ab interaction in this assay is such that the curve describing the relation of absorbances and different dilutions has a sigmoidal form with a negative slope. Hence, the calculated sensitivity value does not sit in the range of suitable precision and accuracy. For that reason, we defined sensitivity in the linear range, and the limit of detection of our ELISA is simply equal to the value of the lower limit of linearity.

Accuracy

The **accuracy** of an experimental method shows the degree of congruence of the experimental results obtained with real (reference material) or expectable value (theoretically estimated from the fitted curve) (Biddlecombe et al., 1996). Accuracy corresponds to the percentage that shows the obtained value in relation to the actual value of results.

Accuracy is represented by the absolute and relative errors, and it is established for the entire determined range of the analytical procedure. For calculation of accuracy, it is necessary to determine the expected/obtained ratio for at least three samples, each used in triplicate.

The expected values were determined after fitting the obtained curve and calculated from the curve fit equa-

Table 1. Parameters of the linear regression equation for the range of linearity of ELISA for detection of IgG antibodies specific for A H1N1, A H3N2 and B viral antigens

	Intercept (a)		Slope (b)		Statistics
	Value	Error	Value	Error	R^2
A H1N1	2.2055	0.0532	–0.2389	0.0105	0.98653
A H3N2	2.1823	0.0573	–0.2054	0.0113	0.97903
B	2.1938	0.0525	–0.2348	0.0104	0.98646

Table 2. Absorbance values of the upper and lower limits of ELISA for detection of IgG antibodies specific for A H1N1, A H3N2 and B viral antigens

Antigen	Upper limit / absorbance (nm)	Lower limit / absorbance (nm)	Detection limit / absorbance (sensitivity) (nm)
A H1N1	1.8483	0.3935	0.1074
A H3N2	1.7783	0.5107	0.1050
B	1.7663	0.3468	0.1201

Table 3. Determination of accuracy using inhibitory ELISA

Parameter	A H1N1		A H3N2		B	
	Value	Error	Value	Error	Value	Error
y0	0.4777	0.0259	0.4341	0.0322	0.3151	0.0137
A1	0.7050	0.0358	0.8271	0.0312	0.8918	0.0178
t1	1.2779	0.2257	2.9806	0.3103	1.4486	0.0989
R^2	0.9725		0.9932		0.9958	

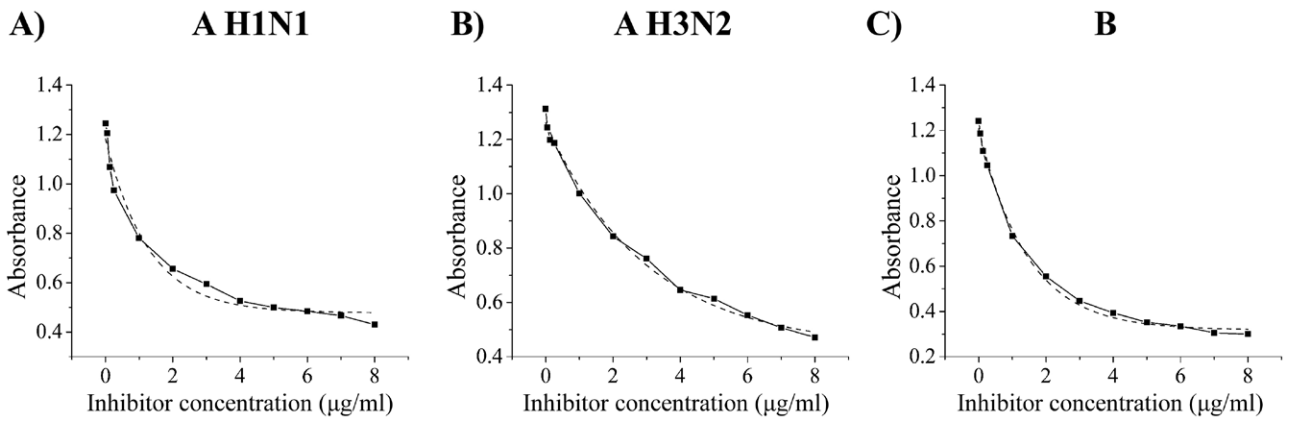

Fig. 5. Estimation of accuracy of ELISA for detection of antibodies specific for whole-virion antigens. The accuracy was estimated by comparison of the experimental (solid line) and expected (dashed line) absorbance values obtained for antigens: A H1N1 (**A**), A H3N2 (**B**), and B (**C**).

tion: y = A1 * exp (–x/t1) + y0 (Table 3). The accuracy was then determined as the ratio between the absorbance calculated from the curve fit (expected absorbance) and the one recorded in the test (obtained absorbance). Accuracy was calculated for 12 concentrations of inhibitors, after which the mean value and standard deviation were determined (Fig. 5). The obtained values of the correlation coefficients R^2 had an extremely high degree of accuracy, which showed that our ELISA was highly accurate. Equation parameters y, A, and t for the fitted curve and correlation coefficients R^2 are shown in Table 3.

Precision

Precision represents the measurement of reproducibility of the recorded results. According to that, the method is more precise if the results obtained in separate tests are similar to each other. Precision is expressed by two parameters: standard deviation (SD) and coefficient of variation (CV) (relative standard deviation). Precision is determined by the following three types of measurements: 1) intra-assay (repetitiveness), which shows that the multiple occurrences of a test sample in the same experiment yield the same result; 2) inter-assay, which shows that the multiple occurrences of a test sample in different experiments (e.g. different days) give the same result; and 3) inter-laboratory assay, which shows that the same pattern is obtained for the same measured value when tested in different laboratories.

Intra-assay precision (repeatability) of ELISA describes how much variation occurs within the assay, and it was calculated based on the value of ten duplicates of the same sample used in one plate. This is the average standard deviation of the mean of duplicates, which are calculated according to the formula: $CV_{intra} = \{[(\Sigma\ SD(d)) : n] : \bar{a}\} \times 100$, where SD(d) is the standard deviation of a duplicate, $\bar{a}$ is the mean of a duplicate sample, and n refers to the number of replicates. The repeatability results obtained for our ELISA are shown in Table 4.

Inter-assay precision describes how much variation occurs between separate assays. The coefficient of variation for **inter-assay precision (intermediate precision)** of our ELISA was determined as the average standard deviation of the mean values of duplicates, according to the formula: $CV_{inter} = \{[(\Sigma\ SD(s)) : N] : \bar{A}\} \times 100$, where SD(s) is standard deviation of all duplicates of the sample from different experiments, $\bar{A}$ is the mean of results obtained for repeated measurements of the same sample, and N represents the number of measurements. The results are shown in Table 5.

According to the literature data (Murray and Lawrence, 1993), the coefficient of variation of 10 % or less for intra- and inter-assay is considered to be satisfactory. The

Table 4. Intra-assay precision (repeatability) of ELISA for detection of whole-virion influenza IgG antibodies

Antigen	$\{[(\Sigma\ SD(d)) : n] : \bar{a}\} \times 100$	CV intra (%)
A H1N1	(0.02843 : 1.81490) × 100	1.566
A H3N2	(0.03458 : 2.00465) × 100	1.720
B	(0.03140 : 1.89830) × 100	1.650

Table 5. Inter-assay precission of ELISA for detection whole-virion influenza IgG antibodies

Antigen	{[(Σ SD(s)) : N] : Ā} × 100	CV inter (%)
A H1N1	(0.02055 : 1.69270) × 100	1.21
A H3N2	(0.04290 : 1.83935) × 100	2.33
B	(0.02486 : 1.71950) × 100	1.44

values of coefficient of variation obtained for our ELISA were under 5 %, which indicates a high precision assay performance for testing samples once or more times.

Inter-laboratory assay precision (reproducibility) refers to the measurement of the same sample in different laboratories. Therefore, it is necessary to determine the average SD of the mean values obtained in different laboratories. We measured inter-laboratory assay precision by performing ELISA with standard antisera in three different laboratories at our Institute. Inter-laboratory CV was calculated by the formula:

$$CV_{inter\text{-}lab} = \{[(SD(1) + SD(2) + SD(3)) : 3] : [(\bar{A}1 + \bar{A}2 + + \bar{A}3) : 3]\} \times 100,$$

where Ā1, Ā2 and Ā3 represent the averages of the results from the first, second and third laboratory, respectively, and SD(1), SD(2) and SD(3) represent corresponding SDs. The results obtained for reproducibility of our ELISA are shown in Table 6. Given that the value of $CV_{inter\text{-}lab}$ is satisfactorily within 10–15 % (Biddlecombe et al., 1996), it can be said that our ELISA is characterized by high reproducibility.

Discussion

The goal of our method was to detect the existence of mouse sera antibodies specific for different epitopes on whole influenza virions induced by immunization and to determine their relative amounts.

Although it has been more than 70 years since it was developed, the HI assay is still the gold standard for serotyping influenza viruses (Schmitz et al., 2015), as well as for determining anti-influenza antibody titres in immunized animals. However, the application of the HI assay can be complicated due to the changes in HA of H3N2 viruses, which have lost their capacity to agglutinate either chicken or turkey red blood cells, both of which are popular choices for use in HI assays (Medeiros et al, 2001). In addition, in order to prepare the fresh erythrocyte suspension for HI, the laboratory animals need to be kept under strict veterinary supervision, which complicates and prolongs the vaccine registration procedure. Considering that the time is crucial for design and control of vaccines in the case of pandemics, we developed a fast and validated ELISA method for detection of mouse sera antibodies specific for influenza virions induced by immunization. This whole virion-based ELISA can be used as a standard anti-influenza antibody assay for pre-clinical testing of newly produced influenza vaccine candidates. This assay fulfils the ICH criteria for precision, linearity, stability, and robustness that are adopted by the regulatory authorities for assay validation.

The critical validation parameters are summarized in Table 7. The specificity of the method was high, and the linearity and scope had correct values. Further, according to the literature data (Murray and Lawrence, 1993), intra- and inter-assay coefficient of variation of 10 % or less is considered to be satisfactory. The values of coefficient of variation obtained for our ELISA were under 5 %, which indicates high-precision assay performance for testing samples once or more times. In addition, given that the value of $CV_{inter\text{-}lab}$ is satisfactorily within 10–15 % (Biddlecombe et al., 1996), our ELISA is char-

Table 6. Inter-laboratory assay precision of ELISA for detection of whole-virion influenza IgG antibodies

Antigen	{[(SD(1) + SD(2) + SD(3)) : 3] : [(Ā1 + Ā2 + Ā3) : 3]} × 100	CV inter-lab (%)
A H1N1	(0.18735 : 1.65155) × 100	11.34
A H3N2	(0.20572 : 1.81323) × 100	11.34
B	(0.21593 : 1.69542) × 100	12.73

Table 7. Summary of validated characteristics of ELISA for detection of mouse IgG antibodies specific for whole influenza virions

Validation characteristics		Antigen A H1N1	Antigen A H3N2	Antigen B
Specificity		High	High	High
Linearity		0.3–1.8	0.3–2.0	0.2–2.0
Range		1.850–0.395	1.780–0.510	1.770–0.350
Accuracy (R^2)		0.9725	0.9932	0.9958
Precision	**Repeatability** (ref. value: ≤ 10%)	1.56 %	1.72 %	1.65 %
	Intermediate precision (ref. value: ≤ 10%)	1.21 %	2.33 %	1.44 %
	Reproducibility (ref. value: 10%-15%)	11.34 %	11.34 %	12.73 %
Detection Limit		0.395	0.510	0.350

acterized by high reproducibility. Compared with other ELISA systems (Murphy et al., 1981; Shafer et al., 1998; Rowe et al., 1999; Moreno et al., 2009; Schlaudecker et al., 2013) developed for detection of influenza-specific antibodies, our assay exhibited superior performance.

We would like to point out that for our validation procedure we used a specific way to determine the degree of accuracy. To calculate accuracy of the assay, defined as the difference between actual (the fitting) and measured values, the values on the *x*-axis must be specific and represented by measurable physical quantity. However, our ELISA will be used for determination of specific antibody titres, which is a relative quantity. Therefore, we performed inhibitory ELISA in order to link the recorded absorbance with a measurable value, specifically, the concentration of inhibitor (Fig. 5). In this way, we were able to measure the accuracy of the method without the use of standard antiserum or a solution with defined antibody concentration. The obtained values of the correlation coefficients R^2 had extremely high degrees of accuracy, which pointed out that our ELISA is highly accurate.

We used this method to determine the relative amounts of anti-influenza antibodies in animal sera immunized with our influenza vaccine candidates. Independently of this, we used our ELISA system for evaluating anti-influenza antibodies in human sera, with proper positive and negative controls. In these tests, the corresponding secondary antibody was anti-human IgG-specific antibody. Although we did not separately validate the human serum ELISA, we were able to conclude that this high-quality method is equally suitable for testing human sera.

Conclusions

In summary, our ELISA for the measurement of mouse serum antibodies specific for whole influenza virions A H1N1, A H3N2, and B was developed and successfully analytically validated. The described assay was used to detect Abs induced after immunization with the influenza vaccine produced in the Institute of Virology, Vaccines and Sera "Torlak". The results of validation are summarized in Table 7 and show that the characteristics of ELISA fit well within the margins that define a good and reliable test. This ELISA is sensitive, safe and highly accurate, and therefore represents a good tool fitting its purpose of analysing the induced antibody response in mice immunized with the influenza vaccine. The method can be used at any moment and will provide reliable data, which is of great importance in the event of a pandemic, when rapid preparation of the appropriate vaccine is needed.

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