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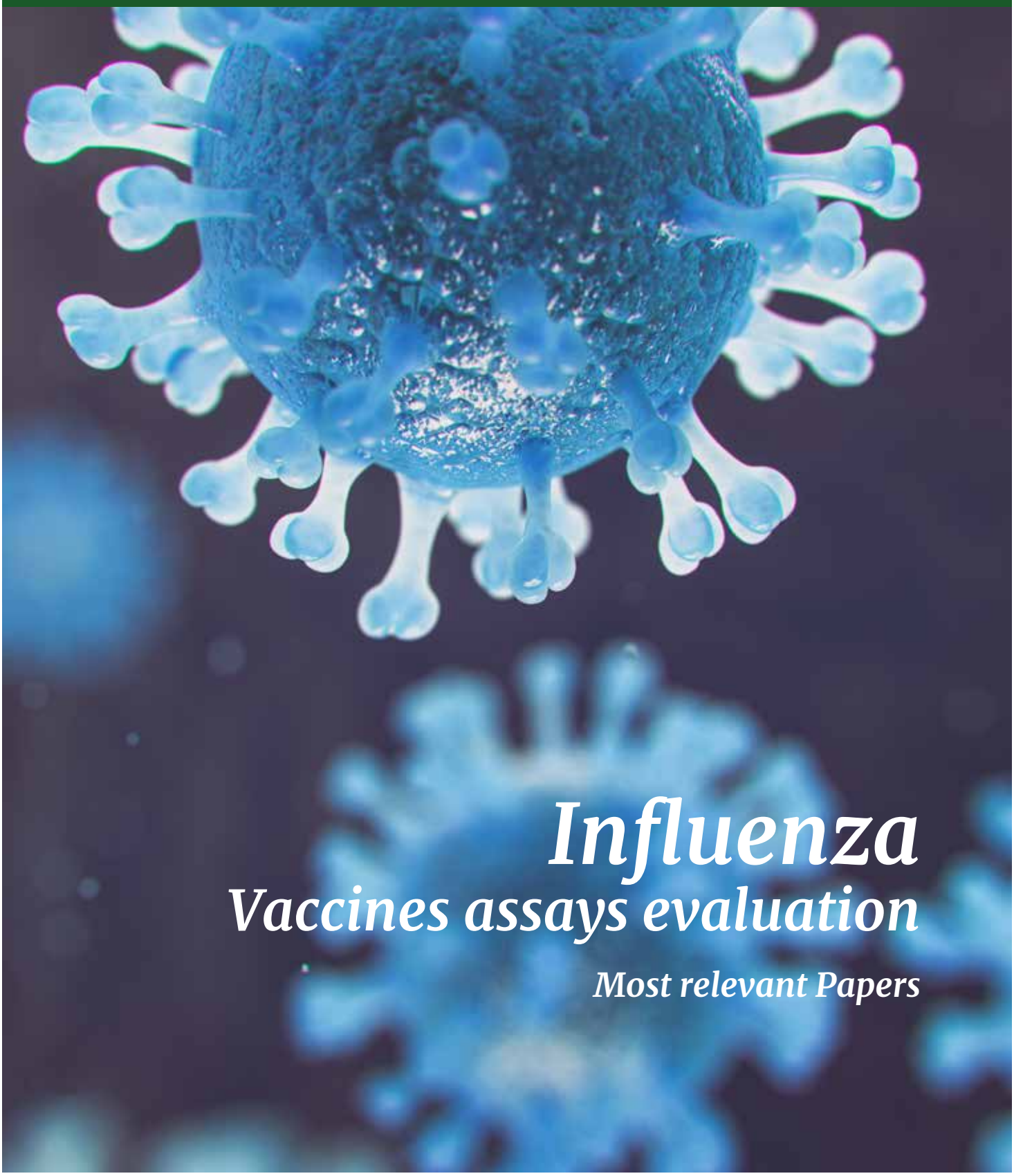

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Harmonization and qualification of an IFN-γ Enzyme-Linked ImmunoSpot assay (ELISPOT) to measure influenza-specific cell-mediated immunity within the FLUCOP consortium

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Influenza continues to be the most important cause of viral respiratory disease, despite the availability of vaccines. Today's evaluation of influenza vaccines mainly focuses on the quantitative and functional analyses of antibodies to the surface proteins haemagglutinin (HA) and neuraminidase (NA). However, there is an increasing interest in measuring cellular immune responses targeting not only mutation-prone surface HA and NA but also conserved internal proteins as these are less explored yet potential correlates of protection. To date, laboratories that monitor cellular immune responses use a variety of in-house procedures. This generates diverging results, complicates interlaboratory comparisons, and hampers influenza vaccine evaluation. The European FLUCOP project aims to develop and standardize assays for the assessment of influenza vaccine correlates of protection. This report describes the harmonization and qualification of the influenza-specific interferon-gamma (IFN-γ) Enzyme-Linked ImmunoSpot (ELISpot) assay. Initially, two pilot studies were conducted to identify sources of variability during sample analysis and spot enumeration in order to develop a harmonized Standard Operating Procedure (SOP). Subsequently, an assay qualification study was performed to investigate the linearity, intermediate precision (reproducibility), repeatability, specificity, Lower and Upper Limits of Quantification (LLOQ-ULOQ), Limit of Detection (LOD) and the stability of signal over time. We were able to demonstrate that the FLUCOP harmonized IFN-γ ELISpot assay procedure can accurately enumerate IFN-γ secreting cells in the analytical

range of 34.4 Spot Forming Units (SFU) per million cells up to the technical limit of the used reader and in the linear range from 120 000 to 360 000 cells per well, in plates stored up to 6 weeks after development. This IFN-γ ELISpot procedure will hopefully become a useful and reliable tool to investigate influenza-specific cellular immune responses induced by natural infection or vaccination and can be an additional instrument in the search for novel correlates of protection.

KEYWORDS

IFN-γ ELISpot, cell-mediated immunity, assay qualification, assay harmonization, influenza

Introduction

Influenza continues to be the most important cause of viral respiratory disease associated with millions of hospitalizations and hundreds of thousands of deaths, despite the availability of vaccines (1, 2). The current seasonal human influenza vaccines are moderately effective in certain populations but require annual updating and administration. Additionally, the vaccine effectiveness varies depending on the match between the vaccine strains and the circulating strains. To overcome these shortcomings, there is an urgent need for new or improved influenza vaccines and efforts are already being made to design long-lasting universal influenza vaccines effective against different variants (3). Today's evaluation of influenza vaccines mainly focuses on the quantitative and functional analyses of antibodies to haemagglutinin (HA) and neuraminidase (NA), the major surface glycoproteins of the virus. Cellular immune responses, primarily mediated by T cells, not only target the mutation-prone surface proteins but also internal proteins that are generally more conserved and shared by heterologous viral strains across influenza A subtypes. Consequently, vaccines inducing cellular immune responses are more likely to elicit broad protection against heterologous viral strains.

Reliable detection and quantification of these cellular responses are of key interest. Therefore, cellular immune assays need to be qualified and, if possible, even validated to demonstrate assay precision, robustness and specificity before being applied in clinical trials. To date, laboratories that monitor cellular immune responses use a variety of in-house procedures. This generates diverging results, complicates interlaboratory comparisons, and hampers influenza vaccine evaluation (4–6). The European FLUCOP project, supported by the Innovative Medicines Initiative Joint Undertaking (IMI-JU, under Grant Agreement 115672), aims to develop and standardize assays for the assessment of influenza vaccine correlates of protection (7). Within this consortium, efforts have been made to develop harmonized Standard Operating Procedures (SOPs) for influenza-specific interferon-gamma (IFN-γ) Enzyme-Linked Spot (ELISpot) and Intracellular Cytokine Staining (ICS) assays, followed by assay qualification. These two cell-mediated immunity (CMI) assays allow for the detection and quantification of antigen-specific cytokine responses to vaccination and infection. The ELISpot assay specifically aims at quantifying IFN-γ producing cells, such as $CD4^+$ Th1 and $CD8^+$ T cells which are the prime subsets of interest when examining influenza-specific responses induced by vaccination and/or infection. Note that other cell populations such as NK- and NK T cells can secrete IFN-γ and contribute to the spot formation in the plate ((8, 9)). Depending on the research questions asked, other cytokines (e.g., IL-2, IL-4, IL-5) (10) can be investigated by ELISpot. The harmonization and qualification of the ICS assay are described in a separate report in this special topic issue (*Begue et al., 2022. Harmonization and Qualification of Intracellular Cytokine Staining to Measure Influenza-Specific $CD4^+$ T Cell Immunity Within the FLUCOP Consortium (submitted)*).

This report describes the harmonization and qualification of the IFN-γ ELISpot assay. First, two pilot studies were conducted to identify sources of variability during sample analysis and spot enumeration with the aim of developing a harmonized SOP. Finally, an assay qualification study was performed to investigate the linearity, intermediate precision (reproducibility), repeatability, specificity, Lower and Upper Limits of Quantification (LLOQ-ULOQ), Limit of Detection (LOD) and the stability of signal over time.

Materials and methods

Samples

Peripheral blood mononuclear cells (PBMC) were isolated from 12 buffy coats obtained from healthy blood donors (Red Cross Flanders). PBMC were also isolated from blood sampled

from 27 healthy volunteers that participated in a clinical vaccine trial that was carried out specifically for these studies. For this, venous blood was collected in heparin-coated blood collection tubes prior to and 7 days after the administration of a seasonal influenza vaccine (alfa-RIX-Tetra© (season 2015/2016 or 2016/2017)). Ethical approvals for this study and the use of blood collected from Red Cross donors were given by the Ethical Committee of the Ghent University Hospital.

PBMC were isolated according to the standardized procedure *FLUCOP SOP for PBMC isolation and cryopreservation*, available as Supplementary Material (Appendix 1). In brief, venous blood samples were diluted 1:2 in Hanks buffered salt solution (HBSS), and buffy coats were brought to a total volume of 300 mL in HBSS. PBMC were isolated by isopycnic centrifugation using Lymphoprep™. Subsequently, cells were washed twice in HBSS, suspended in freezing medium [10% dimethyl sulfoxide (DMSO)/ 90% fetal bovine serum (FBS)], frozen at a concentration of ≥ 5 to ≤ 20 million cells/mL within 24h (buffy coats) or 6h (whole venous blood samples) after blood collection and finally stored in liquid nitrogen until use.

All cryovials were identified with unique codes without any reference to their source. All samples later distributed to other laboratories were selected from this PBMC biobank based on their pre-examined CMI immune responses against influenza antigens.

Pilot studies

Pilot study 1

In pilot study 1, different methods applied to analyse samples were carefully examined. A panel of 24 coded PBMC samples was distributed to 5 consortium partners. Each vial had a unique code allowing only the organizing center to link it with the original specimen identifier. Hereafter, sample identifiers 1 to 24 will be used. Participating laboratories were also provided with antigens for *in vitro* stimulations of the PBMC. These were recombinant Hemagglutinin (HA) H1 A/California/07/2009 (catalog number 3006) and recombinant Hemagglutinin (HA) B/Phuket/3073/2013 (catalog number 3006), both from Protein Sciences Corporation (Swiftwater, PA) and kindly provided by Sanofi. The recombinant proteins were produced in insect cells using the baculovirus expression vector system and purified to at least 90%. Each laboratory was asked to analyze the samples using their in-house procedure for IFN-γ ELISpot and preferred reagents. An online worksheet was filled out to collect information regarding the number of vials thawed, thawing medium, thawing medium temperature, thawing process, FBS validation status, cell counting technique, the technique applied for determination of cell viability, cell resting time and medium as well as the concentration of cells during the resting period (where applicable), culture medium, the use of self- or pre-coated plates, coating antibody, conjugated detection antibody, cell concentration in the well, stimulation/incubation time, substrate for staining, the process of washing the wells, stop reaction, ELISpot reader and related software, Quality Control (QC) process, validation criteria on background conditions, and any comments/deviations that may have occurred. Minimal instructions on data reporting were provided and linked to the lab ID, stimulation condition, mortality percentage, sample ID, investigated marker and counted spots.

Pilot study 2

In pilot study 2, the variation in spot interpretation and data reporting was investigated. An ELISpot plate prepared by one partner (University of Oxford) was distributed to 6 other partners for read-out within 30 days. Storage and transport of this plate were performed at room temperature. Each lab was asked to read the plate shortly after reception according to their in-house procedure, report the data according to minimal instructions provided by the organizing center, and send the plate to the next lab according to the predefined distribution schedule.

Results from both pilot studies and information provided *via* the online worksheets were collected and centrally analyzed by the organizing center (Center for Vaccinology, Ghent University, Belgium). The processed data is reported in a blinded manner in this study.

IFN-γ ELISpot assay procedure and antigen titration

For the pilot studies, the consortium partners were asked to use their in-house procedures and preferred reagents. During assay qualification experiments performed by one FLUCOP partner, the SOP for IFN-γ ELISpot developed by FLUCOP was applied. This procedure is available as Supplementary Material (Appendix 2). Briefly, the FLUCOP IFN-γ ELISpot assay was performed using the human IFN-gamma ELISpot PRO kit (3420-2APW-2, Mabtech). On the first day of the 2-day procedure, the pre-coated plates were re-hydrated by washing 4 times with PBS 1X and were blocked by adding 200 µL culture medium (RPMI supplemented with penicillin/streptomycin, L-glutamine, sodium pyruvate, MEM, ß-mercaptoethanol, FBS). Plates were stored for 2 to 4 hours at 37°C. Meanwhile, the samples were thawed in a water bath at 37°C and washed in culture medium. The cell suspension was centrifuged and cells were washed again in culture medium supplemented with benzonase. After centrifugation, cells were resuspended in culture medium. Cell concentration was measured using a Sysmex hematology analyzer (XN-L-350) and cell viability was determined with propidium iodide (PI; staining of dead cells) using a flow cytometer. After centrifugation, cells were resuspended in culture medium to obtain a concentration of 4 x 10^6 PBMC/mL. The blocking solution was removed from the assay plates and 100 µL stimulation reagent was added to each well. PBMC were plated by adding 200 000 PBMC per well (in 50 µL). The plates were

stored overnight (18-24 hours) in an incubator at 37°C and 5% CO_2. The next day, cell suspensions were removed and plates were washed 5 times with 200 µL lab-grade water per well. Next, 100 µL of detection antibody was added and the plates were incubated for another 2 hours at room temperature. Plates were washed 3 times with 200 µL/well lab-grade water, followed by 3 more washes with 200 µL/well PBS. Substrate (50 µL) was added to each well to visualize the spots. Plates were stored at room temperature and were air-dried in the dark. Spots were enumerated within 7 days after the start of the assay using an automated spot counter (ImmunoSpot® S5, Cellular Technology Limited), followed by manual verification of the identified spots (Quality Control).

The influenza-specific stimulating agent selected for the assay qualification experiments was split A/California (H1N1) virus (batch FA593899), kindly provided by Sanofi. Antigen was titrated to determine the optimal stimulation concentration (Supplementary Figure 1) and investigate any potential cellular toxicity. A final concentration of 1.25 µg/mL was found to be the most appropriate stimulating condition. At this concentration, no cellular cytotoxicity was observed (data not shown) and therefore, this concentration was selected for further PBMC stimulations in ELISpot assays. In some qualification experiments, tetanus toxin (T3194-25UG, Sigma-Aldrich) was used as a control antigen to stimulate the cells *in vitro* at a final concentration of 2 µg/mL. The mock or background condition consisted of PBMC cultured in medium only. Each condition was tested in triplicate and mean responses were reported.

Data analysis

Results from the IFN-γ ELISpot assay were expressed as Spot Forming Units (SFU) per million PBMC and reported as the mean of triplicates, duplicates or as singletons, depending on the lab and/or test condition. For the evaluation of antigen-specific responses, background-subtracted results were used. Negative values were corrected to 1 SFU/10^6 cells. Results from the pilot studies and the specificity experiment were log-transformed prior to further calculations. Individual lab results were compared to the geomean, descriptive statistics were performed and a Z-score was attributed.

The Z-score expresses the relationship of a reported value to the mean of a group of values in terms of standard deviations and shows where that result is positioned in the distribution of all reported values. The Z-score is therefore a good tool to assess the performance of an individual lab within a group. Still, it does not give information on the total imprecision of the group data. A Z-score of 0 indicates that the reported result is identical to the mean result. A Z-score of 1 indicates that the reported result differs one standard deviation from the mean. Z-scores can be positive or negative, with a positive value indicating the score is above the mean and a negative score indicating it is below the mean.

$$z = \frac{x - \mu}{\sigma}$$

µ=mean; σ=standard deviation. As an arbitrary rule of thumb, an absolute Z-score ≤1 can be considered as excellent, a Z-score be-tween 1 and 2 as acceptable and a Z-score >2 should be considered as poor performance requiring further investigation.

Results

Pilot study 1

In pilot study 1, a large variation was already observed in the unstimulated conditions (Figure 1). Two out of 5 labs reported background responses exceeding 50 SFU/million PBMC in more than half of the samples (Figure 1A). This 50 SFU/million PBMC threshold is a commonly applied acceptance or quality criterion (11–13). This large variation is reflected in the Z-scores (Figure 1B) where lab A generally reported higher responses, lab D lower responses, lab C showed a dispersed profile and labs B and E reported values close to the group mean (Z-scores

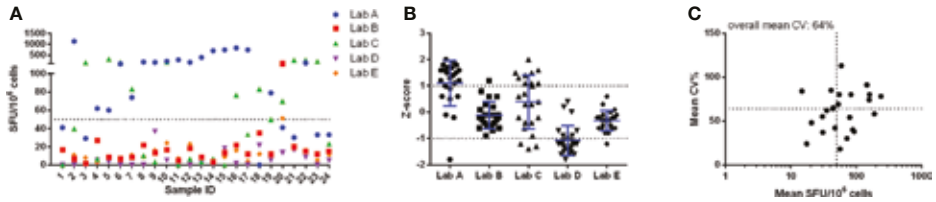

FIGURE 1
Pilot study 1 - unstimulated conditions. Results from each of the 24 samples reported by the 5 labs are given in panel **(A)** and are expressed as SFU/10^6 cells. The performance of the 5 labs is shown in panel **(B)** and is expressed as Z-scores. Panel **(C)** shows the imprecision, expressed as CV%, for the within-sample mean values, expressed as SFU/10^6 cells. Here the horizontal dotted line represents the overall mean CV observed (64%) and the vertical dotted line indicates the arbitrary acceptance criterion of 50 SFU/10^6 cells. Calculations for data shown in panels **(B)** and **(C)** were performed with log-transformed data.

between -1 and 1). The overall mean coefficient of variation (CV) of the background responses was 64% (Figure 1C).

Significant variation was also observed in the antigen-stimulated cultures. The Z-scores demonstrated that all labs reported values relatively close to the group mean, in other words, no lab generated data deviating with more than 2 standard deviations from the group mean. However, the imprecision, expressed as the mean CV, of cultures stimulated with A/California and B/Phuket demonstrated high levels of variation, with 63% and 34% CV, respectively (Figure 2). The lower mean CV upon stimulation with B/Phuket can be explained by the higher range of responses observed, 48.9-2069 SFU/million PBMC, whereas A/California elicited responses in the range of 8.3-319.9 SFU/million PBMC.

Pilot study 2

In pilot study 2, the variation that can be induced upon the enumeration of IFN-γ secreting cells by interpreting developed spots was examined. Figure 3 shows that the Z-scores of the reported counts varied between -1 and 1 (panel A) and that the highest CV values were observed in the lower part of the analytical range (< 5 SFU/well or 25 SFU/million PBMC; panel B). Although limited variation was observed in the enumeration of the spots (mean CV of 25.8%), high diversity in reporting conditions with spots too dense to count was noticed. Some labs reported these particular conditions with a symbol (* or °), one lab with a code ("-2"), one lab with an abbreviation (TNTC, too numerous to count) and two labs with zero ("0"). This observation indicates that careful thought should be given to how such a condition should be flagged and reported. Especially the zero ("0") or "-2" codes can lead to misinterpretation when evaluating (background-subtracted) results as this will still generate a (negative) number. It is important to clearly distinguish non-numerical values (e.g. in case of too dense spots preventing accurate counting) from numerical results (i.e. actual data). A harmonized reporting code needs to be defined.

Establishment of the FLUCOP SOP

Based on information collected *via* the online worksheet (pilot study 1) and a thorough comparison of all applied procedures, a set of potential critical parameters was identified. These were: the cell count technique, application and duration of a cell resting phase, the use of self- versus pre-coated plates, the concentration of cells in the wells, the time of incubation, the medium used during stimulation, the wash step, the ELISpot reader and the FBS validation status (Table 1).

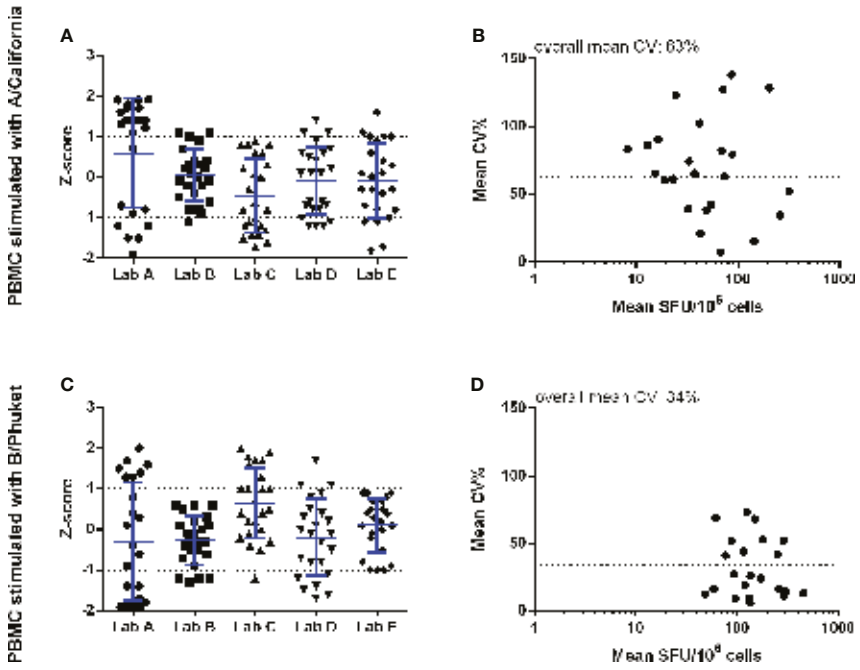

FIGURE 2
Pilot study 1 – Background-subtracted results from the cell cultures stimulated with inactivated split A/California (panels **A, B**) and B/Phuket (panels **C, D**). The performance of the 5 labs is shown in the left panels and is expressed as Z-scores. The observed imprecision, expressed as CV%, for the mean within-sample values, expressed as SFU/10^6 cells, is shown in the right panels. Here the dotted horizontal line indicates the overall mean CV of 63 and 34% for A/California and B/Phuket, respectively. Analyses were performed with log-transformed counts.

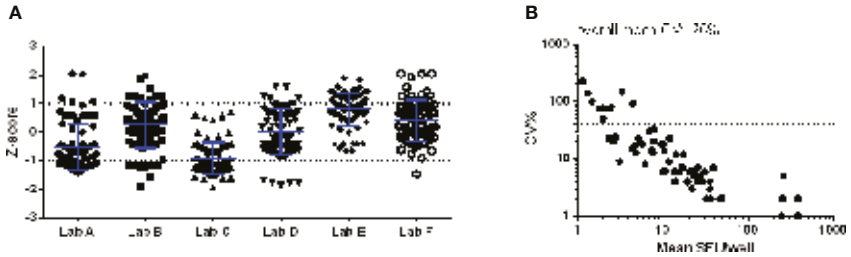

FIGURE 3
Pilot study 2. An IFN-γ ELISpot plate with 84 wells to be scored was distributed to 6 laboratories within one month after preparation. Z-scores per well per lab **(A)** and the CV per well **(B)** are shown. The dotted line in the right panel indicates the CV of 40%, commonly applied as a threshold of acceptable variation in CMI assays.

Within the consortium, a consensus was reached to define a set of specific parameters that had to be strictly applied in the standardized operating procedure for IFN-γ ELISpot testing, whereas for other parameters only a recommendation was proposed (Appendix 2).

IFN-γ ELISpot assay qualification

Linearity

Linearity of an assay is its ability to provide test results that are directly proportional to the concentration of the measurand (quantity to be measured) in a test sample. A standard approach to assess the linearity of a laboratory method consists of diluting a test sample in a negative sample matrix to demonstrate then the assay's ability to reproduce the initial result after recalculation of the result obtained after dilution. In complex cell-based assays, where no reference materials are available, this approach requires the availability of non-responding cells to dilute the cell(s) of interest. In the IFN-γ ELISpot assay, where *in vitro* stimulation occurs immediately prior to the read-out and in the same final reaction vessel, this would require that the diluting cells do not induce any allogeneic or bystander effect on the "tested cells". As this cannot be achieved, an adapted method was designed.

The linearity of the IFN-γ ELISpot was assessed using 4 PBMC samples that were independently fractionally diluted in culture medium without (background condition) or with split A/California virus as stimulating antigen. This was done in 3 replicates and repeated 3 (sample 4) or 4 (samples 1-3) times.

TABLE 1 Overview of the parameters that were identified as potentially critical for the outcome of the IFN-γ ELISpot assay.

Lab ID	Cell count technique	Cell resting	Duration of cell resting (hours)	Plates	Concentration of cells in wells	Incubation time (hours)	Culture medium used in cell resting and during stimulation	Wash step	ELISpot reader	FBS validation
Lab 01	Automated	Yes	4	Self-coated	200.000	18	cRPMI	Automated	AID-ISpotSpectrum	Validated
Lab 02	Automated	No	No	Self-coated	500.000	22	cRPMI	Manually	ImmunoSpot S6, CTL	Validated
Lab 03	Automated	Yes	19	Pre-coated	300.000	24	cRPMI	Manually	ImmunoScan, CTL	No info
Lab 04	Manual	Yes	3	Self-coated	250.000	19	cRPMI	Manually	ELISpot 7.0 - iSpot	Validated
Lab 05	Automated	Yes	1,5	Pre-coated	200.000	19.5	cRPMI	Manually	ImmunoSpot S6, CTL	No
Consensus protocol	**Validated method**	**Yes**	**3**	**Free of choice**	**200.000**	**Overnight (18-24 h)**	**Complete RPMI (cRPMI)**	**Your validated method**	**Your validated equipment**	**Validated FBS**
Fixed or as recommended	Recommended	Fixed	Fixed	/	Fixed	Fixed	Recommended	Recommended	Recommended	Fixed

Fractional dilution of the samples was done by plating 40 000 to 400 000 cells per well. The microculture with 200 000 cells per well was selected as the reference condition for calculating the recovered response.

At this condition of 200 000 cells per well, 127.8 – 15.3 – 15.5 and 13.6 SFU/well were counted in samples 1, 2, 3 and 4, respectively (Table 2). Expressed as SFU per million PBMC, this translated into 639.2 – 76.7 – 77.5 and 67.9 SFU for samples 1, 2, 3 and 4, respectively.

As a rule of thumb, a recovery value between 50 and 150% of the observed reference value, or in other words the ability to detect a decrease or increase of the signal with 50%, is regarded as "linear". Table 2 clearly shows that the responses were linear within the assay conditions ranging between 120 000 cells and 360 000 cells per well. Within this technical assay range, the lowest background-subtracted response observed was 3.5 SFU/80.000 cells. The proportionality of the method was further assessed by calculating the ratios of the number of plated cells versus the observed number of spots per well. Two variables are considered proportional if their corresponding elements have a constant ratio, which is called the coefficient of proportionality. The correlation curves demonstrated high correlations in all 4 measured samples with R^2 values ranging from 0.9734 to 0.9916 (Figure 4).

Within the linear range of 120 000 to 360 000 cells per well and taking into consideration the acceptable recovery range of 50-150%, the lowest and highest acceptable number of spots per well are 3.5 and 331.3 SFU. If this is extrapolated to the standard condition of 200 000 cells per well, this can be further defined as the Lower Limit of Linearity (LLOL) and Upper Limit of Linearity (ULOL) of the IFN-γ ELISpot assay executed according to the FLUCOP SOP and can be set at 25.4 and 1353.3 SFU per million PBMC, respectively.

Intermediate precision and repeatability

The IFN-γ spot forming responses elicited by A/California and TT antigens were measured in 10 PBMC samples. Each sample was tested in duplicate by 2 operators performing each 2 runs on different days, resulting in 8 measurements per sample. The mean responses ranged from 34.4 to 327.5 SFU/10^6 cells and from 2.5 to 201.5 SFU/10^6 cells after stimulation with A/California and TT, respectively (Figure 5A). The variability was assessed by calculating the CV.

Inter-assay or intermediate precision was assessed as the average CV% of all individual measurements per sample after stimulation with TT and A/California and ranged from 14 to 107%, with a mean of 41%. The intra-assay precision or repeatability was assessed and determined across all mean responses obtained per run and ranged from 0 to 36%, with a mean of 21%. The inter-operator precision was also assessed and determined across all mean responses collected per operator for a particular test condition and ranged from 0 to 60%, with a mean of 12%. The level of variation increased substantially in the lower part of the analytical range of this assay, as shown in Figure 5C.

The Lower Limit of Intermediate Precision (LLOIP) was determined by plotting the mean background-subtracted responses to TT and A/California against the mean inter-assay CV% per sample (Figure 5B). The LLOIP was defined as the lowest value with a CV of 40%. A log-log line was considered as the best-fitted curve with an $R^2 = 0.8729$ and $y=10^{(-0,3820*\log(x))+2,189)}$, where y is the mean CV% and x the mean SFU/10^6 cells per sample. The lowest background-subtracted response with a CV of 40% was determined at 34.4 SFU/10^6 cells.

The Lower Limit of Quantification (LLOQ) was defined as the highest value of the LLOL and LLOP and therefore set at 34.4 SFU per million cells. In the white paper published by Corsaro

TABLE 2 Observed SFU per well at each dilution and recovery.

	Sample 1		Sample 2		Sample 3		Sample 4	
Number of cells plated	**Observed mean SFU/well**	**Recovery (%)**	**Observed mean SFU/well**	**Recovery (%)**	**Observed mean SFU/well**	**Recovery (%)**	**Observedmean SFU/well**	**Recovery (%)**
40 000	7.6	28.1	1.0	35.7	0.4	3.2	1.5	19.2
80 000	26.3	51.2	3.7	53.6	3.2	45.5	3.5	76.9
120 000	58.3	75.5	5.0	51.6	5.1	55.2	6.9	100.4
160 000	89.2	87.4	10.0	69.9	9.0	73.9	8.4	88.1
200 000	**127.8**	**100.0**	**15.3**	**100.0**	**15.5**	**100.0**	**13.6**	**100.0**
240 000	155.9	101.3	17.3	98.2	18.3	99.0	17.0	109.0
280 000	191.4	107.0	21.7	71.4	22.7	106.7	22.4	122.7
320 000	221.8	107.7	27.7	72.9	26.7	112.4	25.0	118.6
360 000	270.7	117.5	30.7	84.7	36.2	143.2	31.3	148.1
400 000	331.3	130.6	38.3	107.1	41.6	148.4	34.9	151.3

Recovery is calculated as the percentage of mean SFU/106 cells observed in the reference condition of 200 000 cells plated per well expressed as SFU/106 cells (indicated in bold).

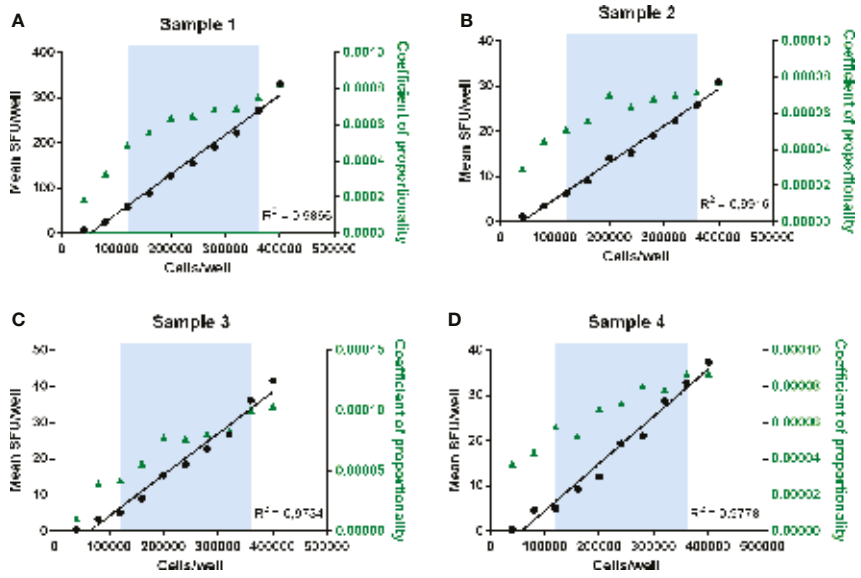

FIGURE 4
Determination of linearity and proportionality. Mean SFU/well is plotted against the number of cells plated per well for each sample **(A–D)**. The blue zone indicates the range between 120 000 and 360 000 cells/well where recovery values ranged between 50 and 150% and therefore linearity was demonstrated. Correlation curves of the number of plated cells per well and the mean responses (SFU/well) are shown in black with the concerned R2 values. The coefficients of proportionality, calculated as the ratios of the observed number of spots per well versus the number of plated cells, are indicated in green triangles and represented on the right y-axis.

et al. (14), the acceptance criterion for the intermediate precision is defined as ≤40% for ≥80% of the samples having mean spot forming units/million cells exceeding the LLOQ. In this study, 96 out of 160 obtained values exceeded the LLOQ determined at 34.4 SFU/million cells, resulting in an intermediate precision of 20% (ranging from 4 to 39%).

Specificity

Assay specificity was determined by stimulating PBMC from 25 paired samples collected before and 7 days after the administration of a seasonal influenza vaccine. PBMC were stimulated with split A/California virus and tetanus toxin, a control antigen unrelated to the vaccine. Specificity was demonstrated if a significant increase in signal was observed between the pre- and post-vaccination samples upon stimulation with the selected influenza-specific antigen ($p < 0.05$) and if no significant increase was observed after stimulation with the control antigen ($p \geq 0.05$). Figure 6 shows a significant increase of the influenza-specific response ($p < 0.001$; Wilcoxon signed-ranked test) and no difference in the TT-specific response ($p = 0.2872$, Wilcoxon signed-rank test).

ULOQ

PBMC from two samples were plated each on one plate. The cells were added to the plates in a serial dilution ranging from 100 000 to 48 cells, with 8 repeats per dilution. All wells were stimulated with the superantigen SEB (staphylococcal enterotoxin B) at a final concentration of 0.25 µg/mL. The Upper Limit of Quantification (ULOQ) is the highest number of counts per well that can be detected and reliably quantified, i.e., a CV% ≤ 40. For both samples, reliable counts were reported up to and including the test condition where 50 000 PBMC were stimulated with SEB with CVs of 4 and 6% Figure 7 (sample 1) and Supplementary Figure 2 (sample 2). The wells seeded with 100 000 PBMC generated too many spots preventing an accurate spot count. The determination of the ULOQ is primarily defined by the instrument and the operator(s) and depends on the availability of strong positive samples. The highest responses that could be enumerated accurately in this analyzing lab were 452.25 and 458.75 SFU/50 000 PBMC or 9045 and 9175 SFU/10^6 PBMC. Based on this information, it can be proposed that samples or conditions resulting in too many spots are reported as '> 10 000 SFU/10^6 PBMC'.

Limit of detection (LOD)

The Limit of Detection (LOD) is the lowest detectable analyte concentration that can be reliably distinguished from analytical noise (15). The technical LOD is calculated as the 95th percentile of the non-specific responses after mock stimulation (medium only). Based on 402 values from the linearity, intermediate precision, repeatability and specificity experiments, the LOD was determined at 12.70 SFU/well or

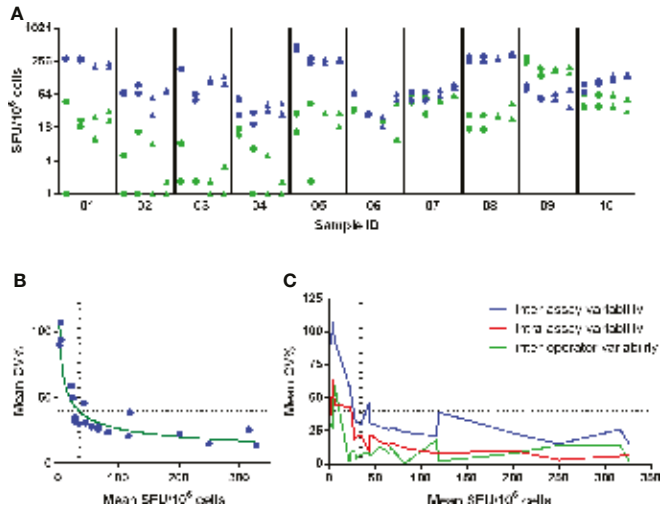

FIGURE 5
Determination of Lower Limit of Intermediate Precision (LLOIP). **(A)** Distribution of background-subtracted responses after *in vitro* stimulation of cells with A/California and tetanus toxin (TT). Each symbol represents a measurement of a certain sample, indicated on the x-axis. A set of 10 samples was tested each in duplicate by 2 operators performing each 2 runs on different days, resulting in 8 measurements per sample. Measurements from operator 1 are indicated with circles and from operator 2 with triangles. Responses obtained after stimulation with A/California are indicated in blue, with TT in green. **(B)** LLOIP was determined by plotting the mean background-subtracted responses to A/California and TT against the mean inter-assay CV% per sample. The LLOIP was defined as the lowest response with a CV of 40% and was determined at 34.4 SFU/10^6 cells. **(C)** The inter-assay, intra-assay and inter-operator variability were assessed by the mean CV% observed for the mean responses of each sample. Each horizontal dotted line represents the cut-off of 40% CV and each vertical dotted line represents the LLOIP determined at 34.4 SFU/10^6 cells.

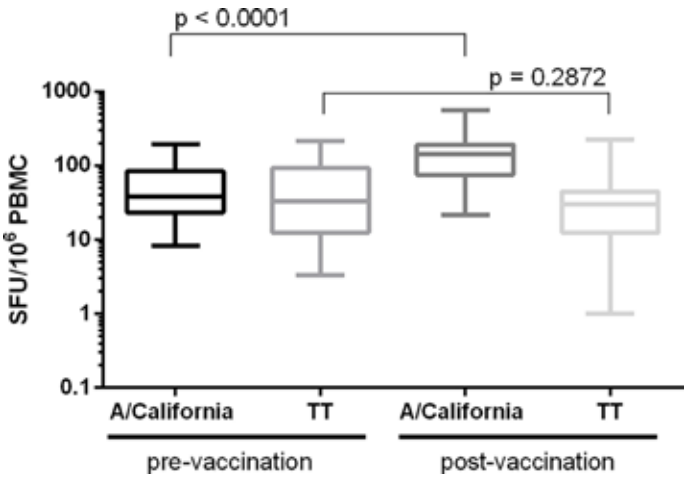

FIGURE 6
Determination of assay specificity. PBMC from 25 paired samples collected before and 7 days after the administration of a seasonal influenza vaccine were stimulated with influenza (split A/California) and control (tetanus toxin; TT) antigens. Background-subtracted values are shown as box plots. Differences in IFN-γ spot forming responses between the pre- and post-vaccination samples following *in vitro* stimulation with either influenza or TT antigen were examined. Wilcoxon matched-pairs signed-rank test was applied and $p < 0.05$ was considered statistically significant.

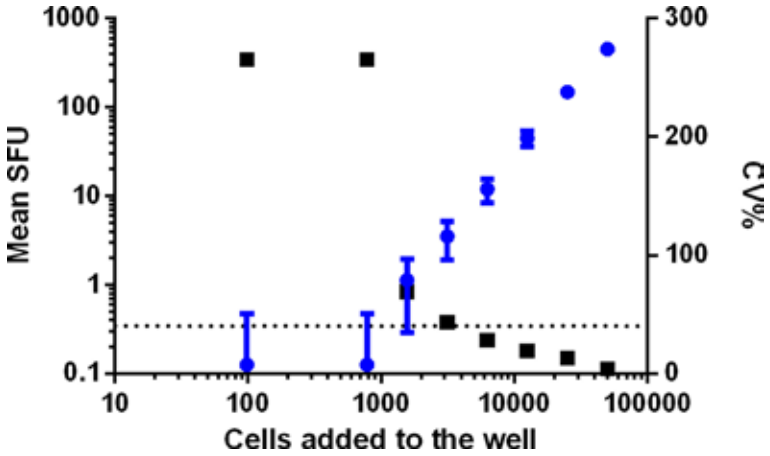

FIGURE 7
Determination of the ULOQ. PBMC from two samples were plated each on one plate in a serial dilution, ranging from 100 000 to 48 PBMC per well. Data from sample 1 is shown. Each condition was repeated 8 times and all cells were stimulated with SEB. Mean SFU with SD bars is indicated in blue and represented on the left y-axis. The related CV% are shown in black and represented on the right x-axis. The dotted line indicates the acceptance criterion of 40% CV.

63.50 SFU/million PBMC. This cut-off could be applied as a criterion of acceptance for background responses instead of the commonly used arbitrary albeit more stringent cut-off of 50 SFU/million PBMC.

Validity of signal stability over time

A set of 9 developed plates was re-read every other week for 24 weeks. The stability of the signal was demonstrated as the counts did not change significantly during this period (Figure 8). The total counts of 96 wells per plate varied with CVs between 2 to 5%, with a decrease of the total sum of counts in 8 out of 9 plates compared to the first read (-1 to -4%) and one plate with an increase of 14%. The absence of striking differences in the reported values for these 3 different operators appears to be an indication of a low inter-operator variability. It is assumed that all involved operators were equally qualified in spot enumeration and because of the limited number of reads per operator this was not further examined. The effect of time was statistically analyzed by performing one-way ANOVA tests. The tests demonstrated a statistically significant effect of time as of week 7 ($p < 0.0001$), meaning that the plates remain stable and can be stored up to and including 6 weeks after development.

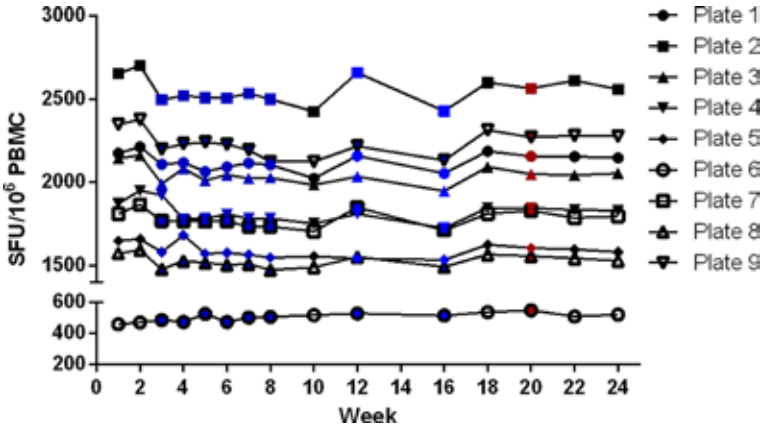

FIGURE 8
Determination of validity of signal stability over time. A set of 9 plates was re-read every other week for 24 weeks. The sum of all antigen-specific IFN-γ ELISpot responses of each plate was evaluated over time. Reading of the plates was performed by 3 different operators, indicated in black (n = 6), blue (n = 8) and red (n = 1).

A summary of all qualification results of the IFN-γ ELISpot assay is shown in Table 3.

Discussion and conclusion

The IFN-γ ELISpot and intracellular cytokine staining (ICS) assays are frequently used to examine cellular immune responses elicited by influenza infection or vaccination (16–21). A better insight into the magnitude, quality and durability of cell-mediated immune responses can improve our understanding of the immunological mechanisms underlying viral clearance or vaccine effectiveness and may contribute to the identification of new correlates of protection. The first prerequisite to generate reliable data is the quality of the samples to be studied. To produce high-quality PBMC, it is desirable to apply a standardized procedure for the isolation and cryopreservation of PBMC that has been proven to be compatible with the envisaged downstream analyses (22–25). Secondly, laboratories that monitor cellular immune responses with the IFN-γ ELISpot assay apply a variety of in-house procedures. This generates diverging results, complicates interlaboratory comparisons, and hampers a reliable evaluation of the immunogenicity of influenza vaccines (4–6). For these reasons, there is still a great need to harmonize the procedures and provide guidance on how to report the assay results in a standardized manner. Within the European FLUCOP consortium, we first developed SOPs for influenza-specific IFN-γ ELISpot (described in this paper) and ICS assays (published in *Begue et al. 2022. Harmonization and Qualification of Intracellular Cytokine Staining to Measure Influenza-Specific CD4$^+$ T Cell Immunity Within the FLUCOP consortium (submitted))* and subsequently performed qualifications of the assays.

Two pilot studies performed in 5 to 6 labs allowed us to identify critical and less critical parameters that can induce assay variation. When every lab applied its in-house procedure, the observed variation, expressed as the coefficient of variation, was 64% in the unstimulated conditions and 63 and 34% in the cell cultures stimulated with split A/California and B/Phuket virus, respectively. Lower variation (mean CV of 25.8%) was observed when only the spot enumeration was assessed. However, high diversity was noticed in conditions where the spots were too dense to count. Based on the reported data and observed ULOQ, '> 10 000 SFU/10^6 PBMC' is proposed as a harmonized reporting code. The overall heterogeneity observed in both pilot studies was considered modest. This may be due to the use of commercial IFN-γ ELISpot kits by several laboratories, a very advantageous possibility not available for all immunoassays. Several assay variables were identified as potential critical parameters: the cell counting technique, the use and duration of a cell resting phase, the use of self- versus pre-coated plates, the cell

TABLE 3 Assay qualification summary.

Assay parameter	Acceptance criteria	Qualification outcome
Intermediate precision and repeatability	The CV for reproducibility should be ≤ 40.% (*The CV for reproducibility should be ≤ 40% for ≥80% of the samples having SFU/10^6 PBMC > LLOQ.) The Lower Limit of Intermediate Precision is defined by the lowest measurement with a CV of 40%.	Stimulation with A/California: Inter-assay CV is 27% (*20%) Intra-assay CV is 10% (*8%) Inter-operator CV is 8% (*8%) Stimulation with TT: Inter-assay CV is 55% (*19%) Intra-assay CV is 31% (*14%) Inter-operator CV is 17% (*6%) Pooled data: Inter-assay CV is 41% (*20%) Intra-assay CV is 21% (*9%) Inter-operator CV is 12% (*7%) LLOIP = 34.4 SFU/10^6 PBMC
Linearity	The range of cell densities (plated cells) through which the recovery was between 50 and 150%.	Linear range: from 120 000 to 360 000 cells/well
LLOQ	The highest value observed of the Lower Limit of Precision and Lower Limit of Linearity.	LLOQ = 34.4 SFU/10^6 PBMC
Specificity	Comparison of pre- and post-vaccination samples: Significant increase after influenza-specific stimulation of the cells ($p < 0.05$) AND Non-significant increase after stimulating the cells with control (non-vaccine) antigen ($p \geq 0.05$)	Stimulation with A/California: $p < 0.0001$ Stimulation with TT: $p = 0.7260$
ULOQ	The highest number of counts per well that can be detected and reliably quantified, i.e. with a CV% ≤ 40.	ULOQ = 458.75 SFU/50 000 PBMC
LOD	The 95th percentile of the responses after mock stimulation.	LOD = 12.70 SFU/well or 63.50 SFU/million PBMC
Signal stability over time	No significant waning of counts over time.	No significant effect of time up to and including 6 weeks after development ($p \geq 0.0001$).

number per well, the incubation time, the culture medium during stimulation, the automated or manual execution of wash steps, the ELISpot reader and the validation status of the FBS. Based on the information collected during the pilot studies, a consensus was reached and a standardized operating procedure for IFN-γ ELISpot testing protocol was developed that consisted of a set of specific parameters that had to be strictly applied, whereas for some additional conditions only a recommendation was proposed. Strict application was required for a cell resting phase (3 hours), seeding density of 200 000 cells per well, an overnight incubation period defined as from 18 to 24 hours and the use of validated FBS. Having a resting phase of cells prior to addition to the ELISpot plate is considered advantageous (26). Cells in an apoptotic state upon thawing will die during the resting phase, and therefore, the proportion of living and good-quality cells will increase leading to a more correct number of plated cells. The number of cells added to a well was also considered a crucial parameter. A single layer of cells is typically achieved by adding 100 000 to 150 000 cells per well. A higher concentration of cells/well can be beneficial for a more effective antigen presentation and co-stimulation. However, an excessively high concentration can lead to spot crowdedness and elevated background spots, which negatively impact the assay sensitivity (27). The latter was observed in pilot study 1 when 500 000 cells per well were seeded by lab A. The consensus was reached to add 200 000 cells/well in the FLUCOP SOP, which is in line with several other publications (28–30). The overnight incubation step was further defined as a period of 18 to 24 hours in line with what the FLUCOP partners were performing. Finally, the use of pre-screened and validated FBS was considered critical because this assay component may cause spontaneous, non-specific cytokine secretion that may have a significant impact on assay sensitivity or may contain toxic factors that can dampen antigen-specific responses.

This harmonized SOP for IFN-γ ELISpot testing was then subjected to a qualification process performed by one FLUCOP partner (CEVAC, Ghent University and University Hospital, Belgium). Unlike for immunoassays such as ELISA, no universally accepted procedures are available to qualify or validate ELISpot assays for regulated use. General criteria that can be applied to define the linearity, intermediate precision, repeatability, LLOQ, ULOQ, LOD and signal stability over time are also lacking. Here we describe the qualification of an IFN-γ ELISpot assay in which human PBMC were stimulated with influenza and tetanus toxin antigens. The acceptance criteria applied to the assay characteristics mentioned above were based on what was available in literature and white papers (14, 15, 28, 29, 31, 32). A summary of all qualification criteria and results is shown in Table 3. The acceptance criterion for reproducibility was set at a CV of ≤ 40%, a cut-off commonly applied in cell-based assays (14, 28). In a first analysis of the data, all values were taken into account to determine the inter-assay variability or intermediate precision. However, a recently published white paper recommended to exclude values below the LLOQ for this calculation (14). These lower counts greatly impact the level of CV because of the higher imprecision in that part of the analytical range of the assay. The observed intermediate precision as of the LLOQ was estimated at 20% and demonstrated the robustness of this assay. The remaining variables responsible for divergent assay results, but more difficult to harmonize, are for example the cell counting techniques and viability measurements, various reagents other than FBS, the ELISpot reader, the spot identification settings that are applied, and the level of experience of the operators. The lack of appropriate reference standards and positive control samples, especially those that mimic test samples, represent additional challenges. Finally, a harmonized protocol itself does not guarantee good performance. Proper execution of a protocol requires skills as well as appropriate training and needs regular quality assessment, not only within a lab but also between labs by conducting interlaboratory or proficiency tests.

Assay qualification is a first step towards assay validation and provides already a means to ensure that the generated data are credible and reproducible. Even in research settings and non-regulated laboratories, this can provide valuable information on the fitness of the assay for the intended use and how to interpret the data (signal versus noise). It is recommended to test a variety of antigens before assay qualification is initiated to define the most suitable stimulating agent compatible with the assay and to answer the appropriate research questions. Furthermore, it is essential that the assay is qualified with the antigen that will be used in the final analysis. If there is a need to change the type of antigen, then a requalification of the assay could be required. However, a change of antigen lot can be supported by performing a bridging experiment without repeating any or all of the qualification experiments, but this should have been addressed by a robustness experiment during assay qualification.

In conclusion, the FLUCOP harmonized IFN-γ ELISpot assay procedure can accurately enumerate IFN-γ secreting cells in the analytical range of 34.4 SFU/million cells up to the technical limit of the used reader and in the linear range from 120 000 to 360 000 cells per well, in plates stored up to 24 weeks after development. We hope this harmonized IFN-γ ELISpot procedure will become a useful and reliable tool to investigate influenza-specific cellular immune responses induced by natural infection or vaccination and will be an aid in the search for novel correlates of protection. We estimate that this harmonized assay may also be applied to cellular immune responses against other (respiratory and non-respiratory) infectious pathogens.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of the Ghent University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FC, GL-R, and GW contributed to the conception and design of the study, data analysis and interpretation, and wrote the first draft of the manuscript. TL, DB, FC, GL-R, and GW contributed to the data acquisition. RC contributed to the critical revision of the manuscript. All authors have read and approved the final version for submission.

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Conflict of interest

Authors EM and EG are employed by VisMederi srl.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.984642/full#supplementary-material

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METHODS
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Validation of a Harmonized Enzyme-Linked-Lectin-Assay (ELLA-NI) Based Neuraminidase Inhibition Assay Standard Operating Procedure (SOP) for Quantification of N1 Influenza Antibodies and the Use of a Calibrator to Improve the Reproducibility of the ELLA-NI With Reverse Genetics Viral and Recombinant Neuraminidase Antigens: A FLUCOP Collaborative Study

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Current vaccination strategies against influenza focus on generating an antibody response against the viral haemagglutination surface protein, however there is increasing interest in neuraminidase (NA) as a target for vaccine development. A critical tool for development of vaccines that target NA or include an NA component is available validated serology assays for quantifying anti-NA antibodies. Additionally serology assays have a critical role in defining correlates of protection in vaccine development and licensure. Standardisation of these assays is important for consistent and accurate results. In this study we first

validated a harmonized enzyme-linked lectin assay (ELLA)- Neuraminidase Inhibition (NI) SOP for N1 influenza antigen and demonstrated the assay was precise, linear, specific and robust within classical acceptance criteria for neutralization assays for vaccine testing. Secondly we tested this SOP with NA from influenza B viruses and showed the assay performed consistently with both influenza A and B antigens. Third, we demonstrated that recombinant NA (rNA) could be used as a source of antigen in ELLA-NI. In addition to validating a harmonized SOP we finally demonstrated a clear improvement in inter-laboratory agreement across several studies by using a calibrator. Importantly we showed that the use of a calibrator significantly improved agreement when using different sources of antigen in ELLA-NI, namely reverse genetics viruses and recombinant NA. We provide a freely available and detailed harmonized SOP for ELLA-NI. Our results add to the growing body of evidence in support of developing biological standards for influenza serology.

Keywords: influenza, enzyme-linked lectin assay (ELLA), neuraminidase inhibition (NI) assay, serology, standardization

INTRODUCTION

Haemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of influenza viruses. Both recognize sialic acid (SA) playing different roles, the HA binds to SA on the host cells allowing virus entry, while the NA has enzymatic activity, removing SA and facilitating the release of progeny virus (1, 2). Currently, 18 HA and 11 NA subtypes have been identified, only a subset of which has been reported in human influenza virus infections.

Since the HA represents the primary target of the antibody response and correlates of protection have been established, vaccine immunogenicity is mainly evaluated on HA specific antibody response (3–5). However, NA inhibiting (NI) antibodies seem to have an independent role in protection, not associated with the prevention of infection, but contributing significantly to immune protection by reducing the severity and duration of infection and by curbing viral shedding and transmission (6). NI antibody titres have been shown to be an independent correlate of protection against influenza disease severity (7, 8). The great advantage of targeting NA is its slower antigenic evolution (9) and the ability to induce longer lasting immunity and cross-protection than that provided by conventional HA-based vaccines (7, 10–12). Some factors such as the immunodominance of the HA, the lack of regulated NA content in vaccine composition and of standardised assays have hindered the study of NI antibodies (5, 7, 13). In 2008, the World Health Organization highlighted the need to further study the role of NA and to develop simpler and more reproducible assays for detecting NI antibodies (14). In 2016, the European Medicines Agency updated the regulatory guidelines on influenza vaccines to include the possibility of evaluating NI antibodies (15).

Currently the most common and widely used technique to evaluate NI antibodies is the enzyme-linked lectin assay (ELLA), originally developed by Lambré et al. (12, 16). The assay is based on the ability of NA to cleave SA residues from a substrate, usually fetuin coated on the surface of 96-well plates. Removal of SA exposes a galactose residue, which is bound by a lectin [peanut agglutinin (PNA)] conjugated to horseradish peroxidase (HRP). The measured optical density (OD) is proportional to the NA activity in the tested samples. The ELLA-Neuraminidase Inhibition (ELLA-NI) titre is defined as the highest serum dilution that shows at least 50% inhibition of the NA activity (17, 18). ELLA is more practical than the traditional thiobarbituric acid (TBA) assay. The TBA assay is based on the detection of free SA, but is cumbersome in nature, uses hazardous reagents, and is not suited to high-throughput testing required for serology studies and NA antigenic characterisation during influenza surveillance (18–22). ELLA-NI and TBA NI titres have been shown to correlate well, however ELLA demonstrates higher sensitivity (23).

One crucial aspect of ELLA-NI is the source of NA since antibodies against HA can interfere and non-specifically inhibit NA activity through a proposed mechanism of steric hindrance (24). To avoid this possible interference, reverse genetics (RG) viruses with antigenically-mismatched HA subtypes, for which human serum samples have no antibodies, has been used for influenza A viruses (18, 19, 22). Other approaches have been evaluated, such as virus-like particles (25, 26), purified recombinant NA (rNA) using a baculovirus expression system (23) and detergent split wild-type viruses (27). Some of these approaches could be useful alternative sources of NA where mismatched RG viruses are not available (19, 20, 28).

To date ELLA-NI has been assessed in an intra-laboratory study (19) evaluating the reproducibility of the ELLA-NI. This study showed that plate-to-plate variability was minimal, the same plate was highly reproducible, and the assay was subtype specific. A subsequent inter-laboratory study (21) confirmed the assay reproducibility even across different laboratories and highlighted the importance of inclusion of a serum standard for the normalization of the NI antibody titres and reduction of variability in results. In addition, the study showed that the antigen titration is a crucial step before performing ELLA-NI,

and an amount of antigen within the linear range of the titration curve should be used. Currently the ELLA-NI has been used for evaluating NI titres in several clinical influenza vaccine studies (23, 27, 29).

In this study we build upon previous work, developed and validated a consensus SOP in an international study involving 7 FLUCOP partners. FLUCOP (http://www.flucop.eu/) is a joint European project between academia, vaccine manufacturers and public health authorities, supported by the Innovative Medicines Initiative Joint Undertaking (IMIJU) aimed at standardising serological assays and developing common protocols for evaluating influenza vaccines. The goal of the FLUCOP project is to have a direct and evidence-based impact on the quality of regulatory decisions and to provide valid and appropriate serological tools for the future definition of alternative correlates of protection for (novel) influenza vaccines. In this study we present a freely available and detailed SOP for testing serum samples using ELLA-NI. We demonstrated this assay was precise, linear, robust within defined limits across multiple testing laboratories, and had subtype specificity. We show this SOP could be used to test both A, and for the first time B (both Yamagata and Victoria lineage), influenza RG viruses. Additionally we demonstrated that rNA could be used as a source of antigen in the assay, with highly reproducible results between laboratories and antigen sources when a calibrator was used to normalise results.

MATERIALS AND METHODS

Antigens and Recombinant Proteins

RG influenza viruses used in this study are described in the **Table 1**. All viruses were propagated in chicken eggs. B viruses were inactivated using β-propriolactone. For H1N1 NA containing viruses, a combination of H7 and H9 RG viruses were used due to differences in BSL of these antigens within different countries. For this study all viral antigens used were BSL2. Recombinant proteins used as antigen or in competition assays are also listed in **Table 1**. Recombinant Na (rNA) proteins were produced in Chinese Hamster Ovaries (CHO) cells

Generation of Influenza B RG Viruses

The chimeric viruses containing HA of H9 and NAs from influenza B viruses were generated by reverse genetics technique using the pHW2000 plasmid as described earlier (30). The HA of these strains is a chimeric protein consisting of the HA ectodomain from H9N2 strain A/Chicken/Beijing/2/97 (H9N2), and the CT+TM (cytoplasmic tail + transmembrane region) from seasonal H1N1 strain A/Brisbane/59/2007. The NA of these viruses are also chimeric proteins containing an ectodomain (stalk and head) of the influenza B viruses (B/Brisbane/60/2008 or B/Phuket/3073/2013) and CT+TM from seasonal H1N1 strain A/Brisbane/59/2007 (**Supplementary Figure S1**).

Clinical Serum Samples

For the end of run study, participating laboratories were asked to select their own panel of 6 in-house human serum samples for testing. For Precision studies a panel of 9 post-vaccination human serum samples and for Linearity a panel of 4 post-vaccination human serum samples were provided to each participating laboratory by Sanofi Pasteur (2015-2016 trivalent influenza vaccine (TIV) (A/California/07/2009, A/South Australia/55/2014, B/Phuket/3073/2013) or 2015-2016 quadrivalent influenza vaccine (QIV) (A/California/07/2009, A/South Australia/55/2014, B/Phuket/3073/2013, B/Brisbane/60/2008)). For testing Robustness and testing B viruses/rNA, a panel of 12 and 16 pre and post-vaccination human serum samples respectively were provided to each participating laboratory by the University of Ghent (Flucop_QIV clinical trial, Fluarix Tetra vaccine containing the following influenza strains: A/Michigan/45/2015 (H1N1) pdm09, A/Hong Kong/4801/2014 (H3N2), B/Brisbane/60/2008 and B/Phuket/3073/2013). Prior to the studies, serum samples were pre-screened in ELLA-NI and selected to cover the dynamic range of the assay. All sera were heat inactivated at 56°C for 1 hour. Serum minus IgA/IgM/IgG (human) was used as a negative control (Sigma-Aldrich S5393).

For the HA competition analysis, 9w-old Female BALB/cByJ mice (Charles River - 327 impasse du domaine Rozier, 69210 Saint-Germain-Nuelles, France) were immunized twice at D0 and D28 and blood samples collected at D49 were pooled. Three mouse sera were tested: a pool of sera from mice vaccinated with monovalent

TABLE 1 | Reverse Genetics (RG) viruses and recombinant proteins use in the study.

Antigen	HA strain	NA strain
RG viruses		
A/H7N1	A/Equine/Prague/56 (H7N7)	A/California/07/2009 (H1N1)
A/H9N1	A/chicken/Beijing/2/97 (H9N2)	A/California/07/2009 (H1N1)
H9-NB/Brisbane	A/chicken/Beijing/2/97 (H9N2)	B/Brisbane/60/2008 (Victoria lineage)
H9-NB/Phuket	A/chicken/Beijing/2/97 (H9N2)	B/Phuket/3073/2013 (Yamagata lineage)
Recombinant proteins		
rHA	A/California/07/2009 (H1N1) influenza (Protein Sciences)	
gB CMV (Sanofi Pasteur)		
rNS1 (JEV, The Native Antigen Company)		
rNA (N1) Tetrabrachion folder		A/Belgium/145-MA/2009 (H1N1) (ThermoFisher Scientific)
rNA (N2) Tetrabrachion folder		A/Hong Kong/4801/2014 (H3N2) (ThermoFisher Scientific)
rNA (B Victoria) Tetrabrachion folder		B/Brisbane/60/2008 (Victoria) (ThermoFisher Scientific)
rNA (B Yamagata) Tetrabrachion folder		B/Phuket/3073/2013 (Yamagata) (ThermoFisher Scientific)

H1N1pdm09 vaccine (monovalent A/California/07/2009, Sanofi Pasteur; sera positive for H1 and N1 antibodies, Haemagglutination Inhibition assay (HAI) titre 160); a serum from a mouse inoculated with rHA (A/California/07/2009) (positive for H1 antibodies, HAI titre 640) and a pool of sera from mice inoculated with PBS (negative for H1 and N1 antibodies).

For specificity, monospecific sera from six individual ferrets infected with wild type (WT) influenza viruses (2 ferrets with A/California/07/2009, 2 ferrets with B/Brisbane/60/2008 and 2 ferrets with B/Phuket/3073/2013) were tested (4 ferrets from Highgate Farm, Male, ages 6 months, 6 months, 9 months and 5 ½ months and 2 ferrets from B&K Marshalls, Male, ages 8 months). Prior to testing sera were heat inactivated at 56°C for 1 hour, receptor destroying enzyme (RDE) treated with 1:10 dilution of the manufacturer's recommended volume of RDE (Denka Seiken, Japan) overnight at 37°C and heat inactivated for 8 hours at 56°C to remove RDE activity.

Participating Laboratories for ELLA-NI Testing

7 laboratories participated in the ELLA-NI studies; in alphabetical order, unrelated to the assigned laboratory codes shown in the Figures and Tables: Abbott, NIBSC, Paul Ehrlich Institute, Public Health England, Sanofi Pasteur, University of Bergen, University of Siena. GSK contributed to design of experiments.

FLUCOP Harmonized ELLA-NI Protocol

Each laboratory received a comprehensive workbook on ELLA-NI testing conditions specific for each study. This described the experimental design for testing linearity, precision, robustness and specificity as well as a detailed SOP for the ELLA-NI including a data reporting template. Sample sera were heat inactivated prior to testing. First, a standard curve of NA activity was carried out for each antigen and used to calculate the dilution required to give 90% of the maximum signal: antigen was serially diluted in PBS and added to a fetuin coated plate. Plates were incubated at 37°C overnight. Plates were washed and lectin from Arachis hypogaea (peanut) (PNA) peroxidase conjugate added and incubated for 120 minutes at room temperature. Plates were washed, TMB added and allowed to develop for 20 minutes then stopped with 0.5M HCl. Plates were read at 450/650mn. The dilution required to give 90% of the maximum signal was calculated. A back titration was carried out to confirm the 90% signal calculation following the procedure described for antigen titration. Once the antigen dilution was confirmed, sera were tested. Serial dilutions of sera and a fixed amount of antigen were mixed before being added to a fetuin coated plate. Plates were incubated at 37°C overnight. Plates were washed, developed and read as described for antigen titration above. The full FLUCOP ELLA-NI SOP can be found in the **Supplementary Material**.

Statistical Analysis

Precision

The coefficients of variation (CV) for repeatability and for intermediate precision were calculated for each sample using a model one-way-ANOVA with the experimental run as a random factor. CV Repeatability (Rep CV) represents the residual variability corresponding to within-run variability. CV Intermediate Precision (IP CV) represents the total assay variability including repeatability and between-run variability. Precision CVs were calculated by sample, by operator for each laboratory on log10-transformed titres. For each lab, a two-way-ANOVA with the sample and the run as random factors was performed on the log10-transformed titres to compute the overall precision using the Mixed procedure of SAS. The acceptance target for functional assays is an IP CV <50%, in line with classical acceptance criteria for neutralisation assays in vaccine licensure.

Linearity

Linearity was determined through a dose proportionality approach. The dose proportionality was tested assuming a power model (31), where the logarithm of the measured concentrations is linearly related to the logarithm of the dilutions. This method tests whether the slope of results vs. dilution may be considered equivalent to 1 (dose proportionality was accepted if the ratio $(GM_H/L)/(GM_L/H)$ lies within the indicative interval [0.5;2] where H=highest dilution, L=lowest dilution GM=geometric mean). When this is true, linearity is accepted for the whole assay range. When this is false, the range is reduced (lowest value removed, followed if required by the highest value, the two lowest values, the two highest values etc.) and retested until the criterion is satisfied, defining the range for which linearity is accepted.

Robustness

Robustness was assessed through an evaluation of end of run effect and an evaluation of three selected parameters on assay performance using a design of experiment (DoE) approach.

End of Run

Any samples with values reported as <10 were excluded from analysis. Any samples where values were missing or reported as out of range on plates 1-5 were excluded from analysis. A reference titre for each sample was calculated as the median value from the first 5 plates of the 20 plates in the run. The ratio 'Result/Median' was then calculated for each sample and plate. For each plate the overall geometric mean ratio (GMR) across all 6 samples was additionally calculated. GMR is considered acceptable within the indicative interval of [0.8-1.25].

Design of Experiment

Two experimental designs were used to assess the effect of incubation time and temperature on assay robustness, based on the ability of laboratories to test two temperatures simultaneously (design 1) or not (design 2). Design 1: For analysis, the reference condition is 37°C, 20 hours virus incubation and 120 minutes PNA incubation and was tested each day. For design 1 firstly day effect was assessed using the GMRs of the reference condition – where a day effect was present, a reference value for GMR calculations was computed by day, where no day effect was observed, replicates across days were used to calculate a reference value. The reference value for a sample was calculated as the geometric mean titre (GMT) of that

sample tested in the reference condition. GMR was calculated as the ratio between a sample titre in a given condition and the reference value for that sample.

For design 2 the reference condition was not tested in each run as only 1 temperature can be used for each run. All incubation times were tested each day with different temperatures per day. Here temperature and day are therefore confounded and the effect of temperature cannot be assessed independently of day effect. The impact of temperature and incubation times is described using two reference values:

i) reference value is the GMT of the sample tested at 37°C, 20h virus incubation and 120 minutes PNA incubation. Here the effect of temperature is assessed (however it should be noted that a random day effect cannot be excluded)

ii) reference value is the GMT of the sample tested at 20 hours virus incubation and 120 minutes PNA incubation by temperature and day (i.e. a different reference value for 36°C, 37°C and 38°C for each day). Here the effect of incubation times is assessed.

GMR was calculated as the ratio between a sample titre in a given condition and the appropriate reference value for that sample. GMR is considered acceptable within the indicative interval of [0.8-1.25].

Calibration of ELLA-NI Titres

Three mid-range samples were selected as calibrators; sample 6 for the precision data, sample 10 for robustness data and sample 20 V2 (donor 20, visit 2 (V2) post vaccination) for the testing of B virus strains and rNA antigen. For each data set a calibration factor was calculated as the ratio of the calibrator titre in an ELLA-NI run/the global GMT of the calibrator sample (GMT of all times the calibrator sample was tested across all participating laboratories). The calibration factor was then applied to other titres within that lab, run and repeat. The GMR was calculated as the GMT of the lab/overall GMT of a sample across all labs. GMR was calculated before and after calibration. For the precision dataset, using a mixed approach of SAS, a 2-way ANOVA with the sample and lab as random factors was performed on $\log_{10}$ transformed calibrated titres to calculate intra-lab %CV and Reproducibility %CV (intra-lab and inter-lab variation combined). For each data set, %GCV (Geometric Coefficient of Variation) across all labs for a sample was calculated as $(10^{s}-1)x100\%$, where s is the standard deviation of the $\log_{10}$ titres. %GCV was calculated before and after calibration ('overall %GCV' is the median %GCV across all samples in a panel), and the change %GCV statistically assessed using the Wilcoxon matched pairs test.

RESULTS

HA Antibody Interference With ELLA-NI and the Use of Appropriate Antigen

An HA competition analysis was carried out to confirm the role of anti-HA antibodies in false positive ELLA-NI titres. Three mouse sera were tested: H1N1 vaccinated (positive for H1 and N1 antibodies), recombinant HA (rHA) inoculated (positive for H1 antibodies) and PBS inoculated (negative for H1 and N1 antibodies). Sera were pre-incubated with either a rHA from A/California/07/2009 virus (H1N1) or two irrelevant proteins (glycoprotein B (gB) protein from cytomegalovirus (CMV) and non-structural-1 (NS1) protein from Japanese encephalitis virus (JEV)) before titration in ELLA-NI using A/California/07/2009 virus (H1N1).

In the presence of both anti-HA and anti-NA antibodies, a 74% reduction in ELLA-NI titre was observed when anti-HA antibodies were competitively bound to rHA (see **Figure 1**). This reduction was specific to incubation with rHA and absent when irrelevant proteins were used. When only anti-HA antibodies were present, competitive binding with rHA abolished ELLA-NI titre. These results demonstrate the role of HA antibodies in the overestimation of NI titres in this assay, confirming the need to either: use reverse genetics virus with an HA not in circulation in humans; remove HA specific antibodies in sera prior to testing; or use an alternative source of NA (for example rNA or lentiviral pseudotypes).

Validation of a Consensus ELLA-NI Standard Operating Procedure (SOP)

We carried out a review of ELLA-NI protocols used by laboratories within the FLUCOP consortium and developed a consensus SOP based on commonality between protocols, taking into account lab-specific limitations and recommendations based on previous publications optimising ELLA-NI. This detailed SOP can be found in the **Supplementary Material**.

Seven laboratories from the FLUCOP consortium participated in a validation of the ELLA-NI SOP, testing precision, linearity, robustness, and specificity in line with classical acceptance criteria for neutralisation assays used for vaccine evaluation (see materials and methods for experimental

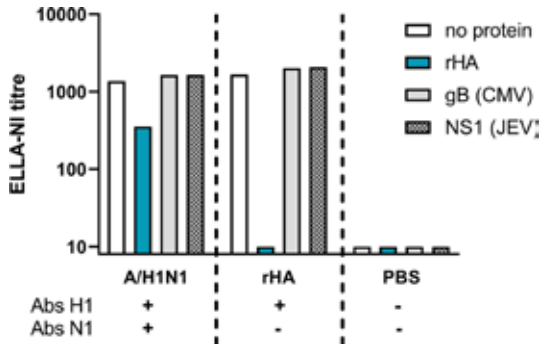

FIGURE 1 | Competitive binding of anti-HA antibodies prior to ELLA-NI testing. Sera from mice vaccinated with A/H1N1pdm09 (A/California/07/2009) monovalent vaccine, rHA (from the same strain) or PBS were pre-incubated either with no protein, with rHA (A/California/07/2009) or one of two irrelevant viral proteins (gB from CMV or NS1 from JEV). Pre-incubated sera were then tested in ELLA-NI with a live H1N1 A/California/07/2009 virus. Sera from H1N1 vaccinated mice contained specific antibodies against both H1 and N1, while sera from rHA vaccinated mice were antibody positive for H1 only and sera from PBS vaccinated mice were antibody negative for both H1 and N1.

design, statistical analysis and acceptance criteria. All testing laboratories used an RG virus containing the N1 NA of the A/California/09/2009 virus with either H7 or H9 (see **Table 1**) for precision, linearity and robustness).

Precision of ELLA-NI

A precision analysis was carried out testing a panel of 9 positive samples spanning the analytical range of the assay. Each sample was tested in duplicate on the same plate, in parallel on a separate plate (giving 2 repeats/operator/run) and by a second operator (Series 1 and 2) on four different days generating up to 16 titres per sample for series 1 and series 2. Six labs participated (5 labs returned data for 2 operators and 1 lab returned data for 1 operator).

Repeatability (Rep) %CV (residual variability corresponding to *within*-run variability) and Intermediate Precision (IP) %CV (total assay variability including repeatability and *between*-run variability designed to mimic routine assay runs) were calculated (see materials and methods and **Table S1**).

For each laboratory the overall IP precision was calculated and was considered acceptable (aiming for an IP %CV <50% for functional assays) ranging from 7.6-34.6% (see **Table 2**). Testing samples in duplicate did decrease IP %CV, however the improvement was small (see **Table S2**) and no clear difference in intra- or inter-plate duplicate IP CVs was observed. Consequently, the routine testing in singleton, where sera volumes are small or to increase throughput, was considered acceptable. Precision by operator was comparable for most laboratories, with some small differences between laboratories in Rep and IP %CV, however IP %CV was still less than 37% across operators and laboratories, below the acceptance target for functional assays of 50% (see **Supplementary Table S3**).

The precision range, delimited by the Lower Limit of Precision (LLP) and the Upper Limit of Precision (ULP), was determined as the range of titres where the IP CV (%) estimated is lower than 50% for each laboratory (see **Table 2**).

ELLA-NI Is Linear Across a Large Titre Range in All Testing Laboratories

An assessment of dilutional linearity was carried out using 4 serum samples diluted ½, ¼ and ⅛ in a negative matrix (an Ig depleted serum – Sigma S5393). Each fractional dilution was carried out independently. Undiluted sera and the three fractional dilutions were tested in 8 runs (4 repeats by 2 operators), except for Lab 5 (4 repeats by 1 operator). All dilutions for a single serum sample were run on the same plate. Linearity was determined through a dose proportionality approach (see materials and methods). All 6 labs demonstrated linearity across the range of samples tested giving the lower limit of linearity (LLL) and the upper limit of linearity (ULL) for each laboratory (see **Table 3**).

Limits of Quantitation

Using the linearity and precision profiles of each laboratory, the limits of quantitation can be defined: the lower limit of quantitation (LLOQ) is the higher value between LLL and LLP, and the upper limit of quantitation (ULOQ) is the lower value between ULL and ULP. **Table 3** gives the LLOQ and ULOQ of the six testing laboratories. LLOQ is consistent between laboratories (min-max 31-64), ULOQ is more variable from lab to lab (min-max 1846-6504) however a large range of titres are within these limits for all testing laboratories.

ELLA-NI Robustness: End of Run Analysis

End of run analysis was designed to identify the maximum number of plates that can be tested in a single assay run. The same set of 6 samples was tested on 20 plates in a single run. Seven laboratories took part in the testing. By laboratory, a reference titre for each sample was calculated as the median titre of the first 5 plates. The ratio 'Result/Median' was calculated for each sample on each plate, and then the geometric mean ratio (GMR) of all samples on one plate was calculated. We expect the GMR of each plate to fall within the indicative range [0.80-1.25]. **Figure 2** plots the GMRs for 7 participating laboratories; two labs provided data for two operators (A and B). Laboratories 1, 3 and 7 had consistent GMRs, however laboratories 2 and 5 showed a systematic bias with GMR decreasing over the 20 plates, laboratory 6A showed a systematic increase in GMR and laboratories 6 and 4 showed an increase in variability in GMR as the number of plates increases. As a conclusion from these results we recommend a limit of 10 plates per run to avoid systematic bias and reduce within-run variability. It should be noted that we did not investigate the impact of including a calibrator on each plate within a run: it is possible that a greater number of plates could be run using this approach. The recommendation of a 10-plate limit applies where a calibrator is not included on each plate.

TABLE 2 | Overall precision analysis - intermediate precision (IP) and repeatability (Rep) %CV per laboratory (acceptance target of IP CV < 50% for functional assays).

			Series 1		Series 2		Precision range	
Lab	N samples	N results used	RepCV (%)	IPCV (%)	RepCV (%)	IPCV (%)	LLP	ULP
1	9	144	10.4	31.6	15.8	34.4	27.1	4855.5
2	8	128*	14.9	24.7	15.4	27.8	30.4	2010.9
3	9	144	10.7	20.3	10.6	15.5	63.8	7555.7
4	9	144	20.2	34.4	20.0	34.6	32.8	7023.5
5	9	72**	9.7	19.6	8.9	16.1	52.3	6901.9
7	9	144	5.5	7.7	4.7	7.6	11.4	3733.9

** Lab 2 returned titres of < 10 for a sample and this sample was excluded from analysis.*

***Lab 5 returned data from 1 operator only.*

TABLE 3 | Summary of LLOQ and ULOQ for each testing laboratory.

Lab	LLP	ULP	LLL	ULL	LLOQ	ULOQ
1	27.1	4855.5	31.4	4202.8	32	4202
2	30.4	2010.9	23.9	1846.9	31	1846
3	63.8	7555.7	62.2	6252.8	64	6252
4	32.8	7023.5	41.9	2238.7	42	2238
5	52.3	6901.9	18.0	6504.1	53	6504
7	11.4	3733.9	41.5	3727.3	42	3727

Lower and upper limits of precision (LLP and ULP), lower and upper limits of linearity (LLL and ULL), and lower and upper limits of quantitation (LLOQ and ULOQ) are shown for each laboratory. LLOQ and ULOQ define the range in which the FLUCOP ELLA-NI SOP delivers both precision and linear accuracy for the testing laboratories.

ELLA-NI Robustness: Impact of Incubation Times and Temperature

Variation in three main parameters was assessed for impact on ELLA-NI titres: virus incubation time (20h +/-1h), virus incubation temperature (37°C +/- 1 degree — or +/- 2 degrees for Lab 1) and PNA incubation time (120 min +/- 15 mins). Laboratories tested 12 samples using two different experimental designs depending on the testing capability of each laboratory. Experimental design 1 was carried out where multiple temperatures could be tested within a single ELLA run (see **Table 4** for experimental design). GMR was calculated using the appropriate reference value for each laboratory for each condition (see materials and methods). Almost all GMRs fell within the indicative interval [0.8-1.25] and no specific condition was associated with changes in GMR (see **Figure 3A**). Experimental design 2 was carried out where a single temperature could be used per ELLA run (see **Table 4** for experimental design). To assess the impact of temperature (**Figure 3B**) and virus and PNA incubation times (**Figure 3C**), appropriate reference values for each sample were computed (see materials and methods) and GMR per lab per condition calculated. As for experimental design 1, almost all GMRs fell within the indicative range [0.8-1.25] and no specific condition was associated with changes in GMR. These data indicate that the assay was robust within the following tolerances: virus incubation temperature 37+/-1/2°C, virus incubation time 20

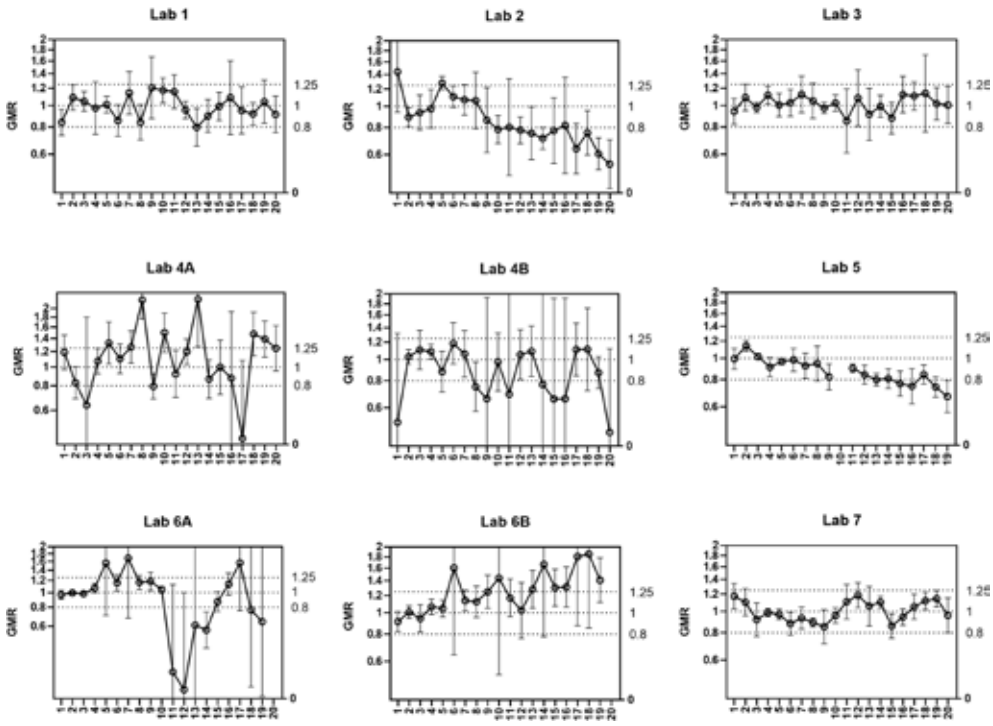

FIGURE 2 | Within-run assay performance – number of plates per run (end of run). 6 samples were tested per plate for 20 plates in a single run. Seven participating laboratories returned data (two labs returned data for 2 operators - A/B) carrying out a single run. A reference titre for each sample was calculated as the median from plates 1-5 for geometric mean ratio (GMR) calculation. The geometric mean of GMR per plate is plotted over 20 plates (error bars show 95%CI). Indicative interval of 0.8-1.25 is shown by dashed lines. After data exclusion as in material and methods Lab1 N=6, Lab 2 N=6, Lab 3 N=5, Lab 4 N=6, Lab 5 N=4, Lab 6 N=6, Lab 7 = 5.

TABLE 4 | Experimental conditions for design 1 and design 2 to assess robustness of ELLA-NI.

Experimental design 1

Condition	Temp (°C)	O/N incubation (hours)	PNA incubation (minutes)
1	(35*)-36	19	105
2	(35*)-36	19	135
3	(35*)-36	21	105
4	(35*)-36	21	135
5	37	20	120
6	38-(39*)	19	105
7	38-(39*)	19	135
8	38-(39*)	21	105
9	38-(39*)	21	135

Experimental design 2

Condition	Temp (°C)	O/N incubation (hours)	PNA incubation (minutes)
1	36	19	105
2	36	19	135
3	36	21	105
4	36	21	135
5	37	19	105
6	37	19	135
7	37	21	105
8	37	21	135
9	38	19	105
10	38	19	135
11	38	21	105
12	38	21	135

**Lab 1 tested a variation of +/- 2 degrees.*

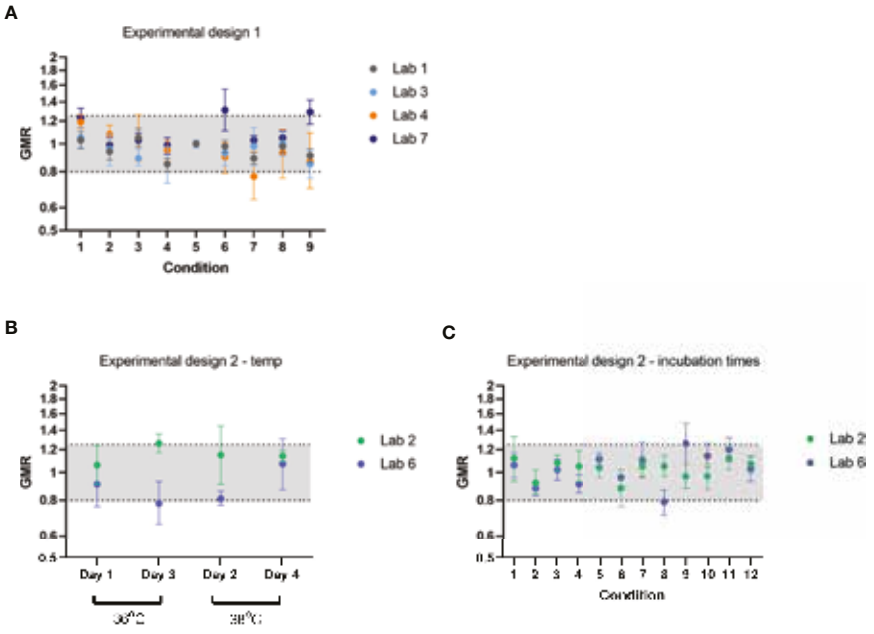

FIGURE 3 | Robustness of FLUCOP ELLA-NI SOP; varying incubation time and temperature. Geometric mean ratios (GMR) per condition across 12 samples are plotted for each testing laboratory. Conditions tested are varying combinations of virus incubation temperature (37°C +/- 1 degree — or +/- 2 degrees for Lab 1), virus incubation time (20h +/- 1h) and PNA incubation time (105/120/135 minutes) according to a design of experiment (see **Table 4**). Two experimental designs were carried out based on the laboratories' ability to test multiple temperatures in a single ELLA run **(A)** or a single temperature per run **(B, C)**. Different statistical analysis approaches were used for the different designs to allow for day effect and confounding factors. GMRs were calculated relative to an appropriate reference value for each sample, for each design, as described in materials and methods. Upper and lower 95% CI are shown as error bars. The indicative range of 0.8-1.25 is shaded in light grey.

hours +/-1h and PNA incubation time 120 minutes +/-15 minutes.

Intra-Laboratory Variability in ELLA-NI Is Comparable Across Influenza A and B Viral Antigens

Two RG influenza viruses were generated containing the NA of B/Brisbane/60/2008 and B/Phuket/3073/2013 (both contain an influenza A H9 HA, see materials and methods and **Supplementary Figure S1**). This allowed us to test mismatched influenza A and B (both Yamagata and Victoria lineage) viruses side by side for the first time. Three laboratories tested three RG viruses: H9 with B/Brisbane/60/2009 NA; H9 with B/Phuket/3073/2013 NA; H7 or H9 with A/California/07/2009 NA against a panel of 16 human sera samples. Laboratory performance for all three virus strains tested was comparable to results from previous testing with N1 alone (see **Figure 4**). Overall %GCVs for samples tested with both B strain viruses were acceptable ranging from 15.8-17.4 for B/Brisbane/60/08 and 15.9-18.6 for B/Phuket/3073/13. Variation in %GCV was uniform across the sample panel tested (see **Supplementary Figure S2**) with no strain specific differences. These results demonstrate the consistent performance of the Flucop ELLA-NI SOP with multiple RG mismatched viral antigens including influenza B viruses.

ELLA-NI Demonstrates Specificity for Different Influenza Types and B Lineages

Specificity studies for influenza serology assays cannot be carried out using human sera, as individuals have a complex immunological history of exposure to multiple influenza strains or vaccines. To overcome this, monospecific ferret sera were used to test the specificity of the ELLA-NI. These ferrets were exposed to single strain of influenza and are negative for antibodies against other influenza strains. Sera tested were from ferrets infected with a) B/Brisbane/60/2008 WT virus (B/Victoria lineage) (2 individual ferrets), b) B/Phuket/3073/2013 WT virus (B/Yamagata lineage) (2 individual ferrets) and c) A/California/07/2009 (H1N1)pdm09 WT virus (2 individual ferrets). These viruses have the same NA as the antigens used in the ELLA-NI. Preliminary studies showed that sera require RDE treatment to remove non-specific inhibitors of NA activity. In agreement with previously published data, RDE diluted to 1:10 was sufficient to remove non-specific inhibitors and a longer 8 hours heat inactivation at 56°C was required to remove all RDE activity (20). Ferret sera were pre-screened using HAI to confirm the absence of anti-H9 or anti-H7 antibodies that could cause non-specific NA inhibition (data not shown). Ferret sera were tested in both homologous (NA serum raised against the same virus as the test antigen) and heterologous (NA serum raised against a virus different from the test antigen) pairs. Type specificity was clear, and ELLA-NI also differentiated between the B Yamagata and B Victoria lineage viruses (see **Supplementary Figure S3**). NI titres for homologous NA/anti-sera pairs were high and reproducible for all three influenza virus strains tested (titres for the 2 individual ferrets for B/Phuket were 2643/4137; B/Brisbane were 2942/3196 and A/California were 1561/1823). Heterologous NA/anti-sera NI titres were very low or negative (range of 5-31, GMT of 8.5).

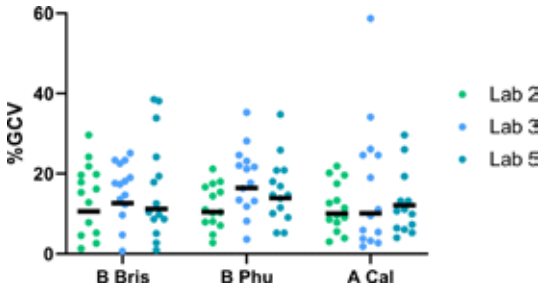

FIGURE 4 | Intra-laboratory performance using three influenza strains is consistent. Three mismatched influenza viruses were tested using the FLUCOP ELLA-NI SOP. A serum panel of 16 human sera was tested in three laboratories. Each laboratory carried out three independent runs. %GCV per sample is shown by lab for: B Bris (H9 with B/Brisbane/60/2008 NA), B Phu (H9 with B/Phuket/3073/2013 NA) and A Cal (H7N1 or H9N1 with A/California/07/2009 NA). The geometric mean of %GCV is shown as a black bar (negative samples 12 and Ig- were excluded from analysis).

The Use of a Mid-Titre Human Serum as a Calibrator Improves Inter-Laboratory Agreement

Using the data obtained in our precision analysis, we selected serum sample 6 (having mid-range titres in all testing laboratories) to calibrate our precision results. A per run calibration factor was calculated and applied to titres. After calibration, the GMR for each sample was calculated (sample GMT by lab/overall GMT across all labs). As expected, the GMRs of calibrated titres were closer to 1 than those of un-calibrated titres, and %GCV was significantly reduced for all samples tested (see **Figures 5A, B**). We used calibrated titres to calculate the Intra-lab CV (reflecting within-lab variability), inter-lab CV and Reproducibility CV (reflecting the total inter-laboratory variation including intra-lab variation). **Table 5** shows the Intra-lab CV, Inter-Lab CV and Reproducibility CV before and after calibration per lab, run and repeat with mid-titre sample 6. The use of a calibrator had little impact on intra-laboratory variation as expected, but substantially reduced inter-laboratory variability with Reproducibility CV reducing from 73% to 30%. These data clearly demonstrate the benefit of using a calibrator to reduce inter-laboratory variation when testing an N1 virus in ELLA-NI. We repeated this analysis using data obtained in the robustness study. Comparison of GMR and %GCV for titres obtained for samples 7-12, tested using the FLUCOP SOP, before and after calibration with mid-range titre serum 10 showed reductions of GMR and %GCV (**Figures 5C, D**).

We next assessed the use of a calibrator using the data from testing of multiple influenza strains. We selected serum sample 20 V02 (having mid-range ELLA-NI titres for B/Brisbane/60/2008, B/Phuket/3073/2012 and A/California/07/2009 RG viruses) as a calibrator. **Figure 6** shows the GMR of ELLA-NI titres by virus strain before and after calibration (**Figures 6A–C**)

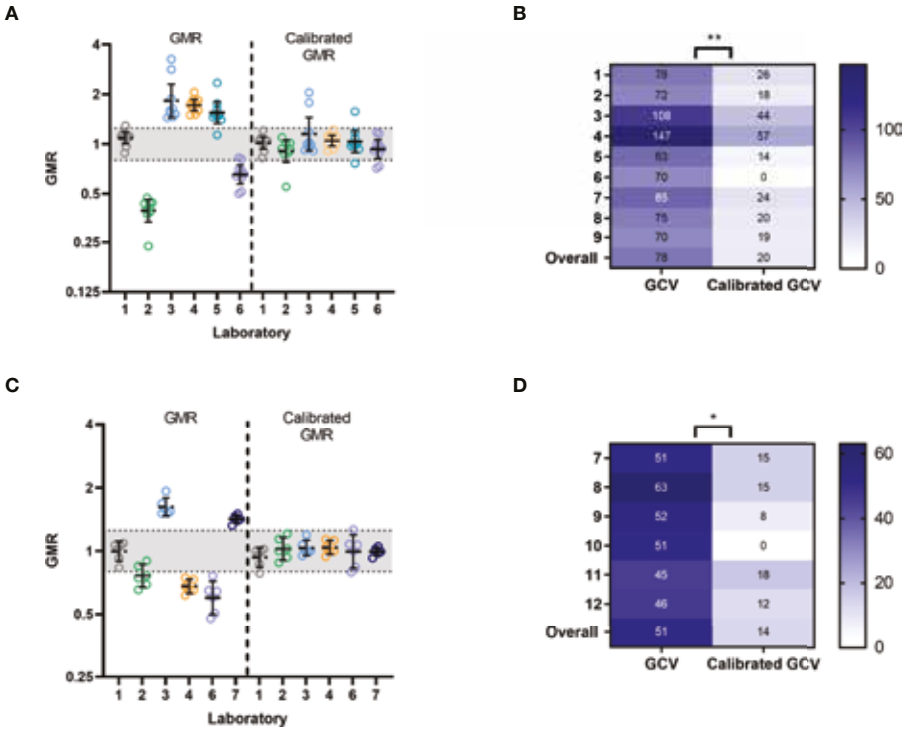

FIGURE 5 | Calibration of Precision data and Robustness data**: (A)** Precision data: geometric mean ratios (GMR) for uncalibrated and calibrated ELLA-NI titres by lab per run. For each laboratory and run, a calibration factor was calculated using sample 6 (see materials and methods, sample with a mid-range titre) and applied to titres. The indicative range of 0.8-1.25 is shaded in grey. Geometric mean and 95% CI error bars are shown in black. **(B)** Precision data: %GCV for log_{10} transformed titres before and after calibration with sample 6. ** indicates significance using the Wilcoxon matched pairs test [P=0.0039] **(C)** Robustness data: GMR of ELLA-NI titres for samples 7-12 are plotted before and after calibration with serum sample 10 (selected as a sample with a mid-range titre). The indicative range of 0.8-1.25 is shaded in grey. Geometric mean and 95% CI error bars are shown in black. **(D)** Robustness data: %GCV for log_{10} transformed titres before and after calibration with sample 10. * indicates significance using the Wilcoxon matched pairs test [P=0.0313].

and inter-laboratory %GCV before and after calibration for each serum sample (**Figure 6D**). A significant improvement in inter-laboratory agreement could be seen after calibration as GMRs were closer to 1, with most values falling within the indicative range [0.8-1.25]. Overall %GCV was significantly reduced for all three virus strains (from 37% to 27% for B/Brisbane60/2008, from 55% to 19% for B/Phuket/3073/2013 and from 40% to 27% for A/California/07/2009). Together these data clearly show the benefit of using a calibrator to reduce inter-laboratory variation in multiple independent studies testing three different strains of influenza.

The distribution of ELLA-NI titres before and after calibration, along with two pre-(V01)/post-(V02) vaccinations pairs included in the serum panel can be seen in **Supplementary Figure S4**.

Evaluation of N1, N2 and B rNA as Source of Antigen in ELLA-NI

We finally evaluated the use of rNA in ELLA-NI using the same 16 sample serum panel tested with mismatched RG N1 and B viruses. Sera were titrated in ELLA-NI against rNAs from 4 influenza strains: A/Hong Kong/4801/2014 (N2), B/Brisbane/60/2008, A/Belgium/145-MA/2009 (N1, A/California/07/2009-like) and B/Phuket/3073/2013, using an MES based buffer. **Figure 7** shows ELLA-NI GMTs. A/Belgium/145-MA/2009 and B/Phuket/3073/2013 were additionally tested using a PBS-based buffer (see **Figures 7C, D**). Titres measured using an MES-based buffer were consistently higher than those measured using a PBS-based buffer: the difference was more pronounced with the rNA from B/Phuket/3073/2013 (**Figure 7D**), with increases ranging from 1.5- to 8.6-fold, than with the rNA from A/Belgium/145-

TABLE 5 | Intra-lab CV, Inter-lab CV and Reproducibility CV for uncalibrated titres and calibrated titres by lab, run and repeat.

Titres	Intra-lab CV (%)	Inter-lab CV (%)	Reproducibility CV (Intra-lab and Inter-lab) (%)
Uncalibrated	24.73	66.48	72.81
Calibrated by lab, run and repeat	23.87	18.45	30.49

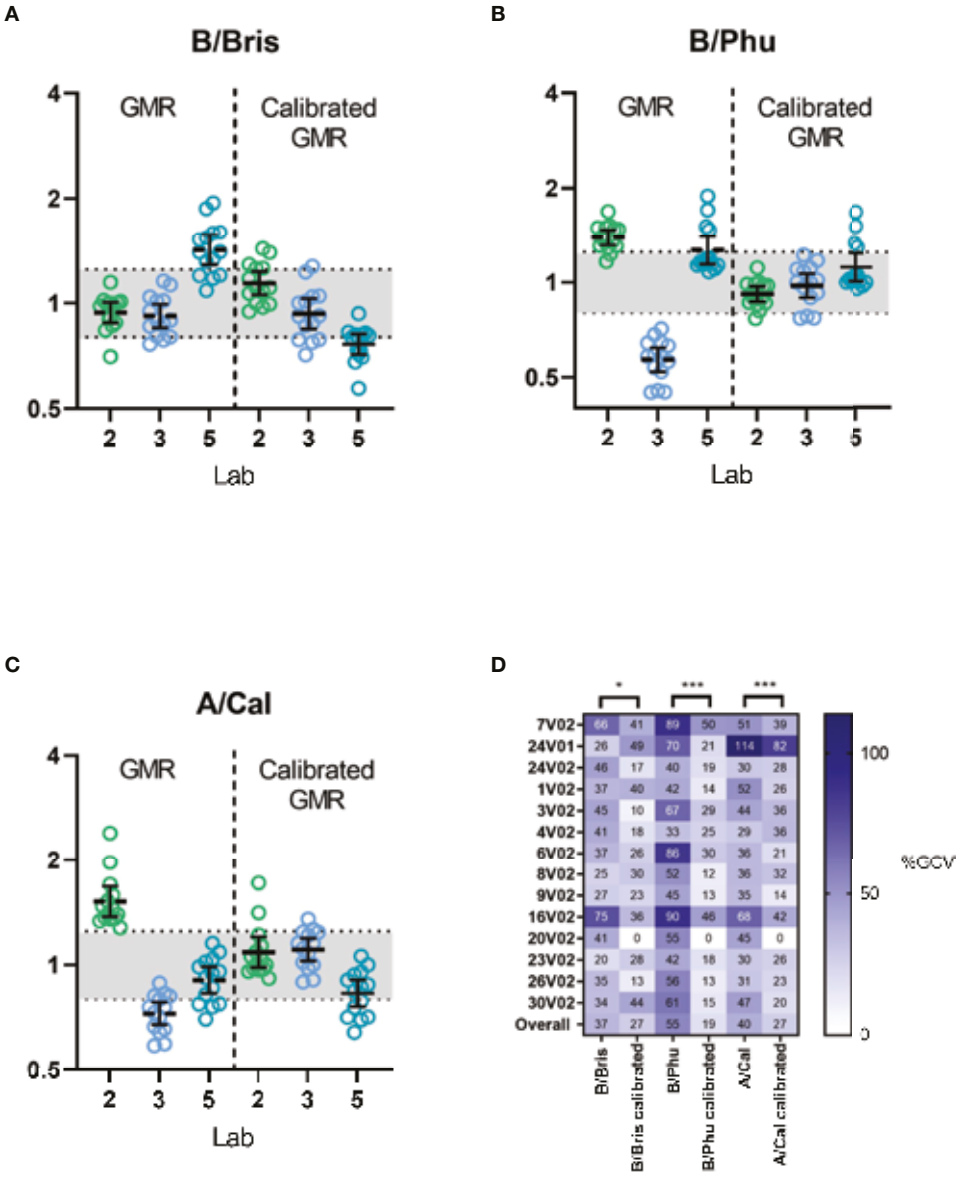

FIGURE 6 | Inter-laboratory performance before and after calibration. Three reverse genetics (RG) influenza viruses, **(A)** H9 with B/Brisbane/60/2008 NA, **(B)** H9 with B/Phuket/3073/2013 NA and **(C)** H7N1 with A/California/07/2009 NA, were tested using the FLUCOP ELLA-NI SOP. Geometric mean ratios (GMR) of ELLA-NI titres are shown before (left) and after (right) calibration using serum sample 20 V02 as a calibrator. The indicative range of [0.8-1.25] is shaded in light grey. The geometric mean and 95% CI error bars are plotted in black. **(D)** %GCV per sample is shown before and after calibration (negative samples 7V01 and Ig- were excluded from analysis). * indicates statistical significance using the Wilcoxon matched pairs test [* P=0.0494, ***P=0.0001 and ***P=0.0009 respectively].

MA/2009 (**Figure 7C**), with increases ranging from 1.3 to 3.0-fold. This strain-strain variation was likely due to differences in NA activity at low (MES buffer) versus neutral (PBS buffer) pH consistent with previous observations (18). As expected, for the two volunteers for which paired sera (pre-and post-vaccination) were available (7 V1/2 and 24 V1/2), titres were increased after vaccination with all the tested antigens. The titre of the Ig depleted human serum negative control was found to be below the detection level in all the tested conditions.

Calibration Using a Mid-Titre Serum Sample Improves Agreement in ELLA-NI Titres Between rNA and RG Virus Antigens

As we previously tested the NA from B/Phuket/3073/2013 both as a mismatched RG virus and as an rNA using the same serum panel, we were able to assess the impact of calibration on agreement in NI titres using these different sources of antigen.

Figure 8A shows the GMTs of B/Phuket/3073/2013 tested in three laboratories using mismatched RG virus (Lab 2/3/5) and the

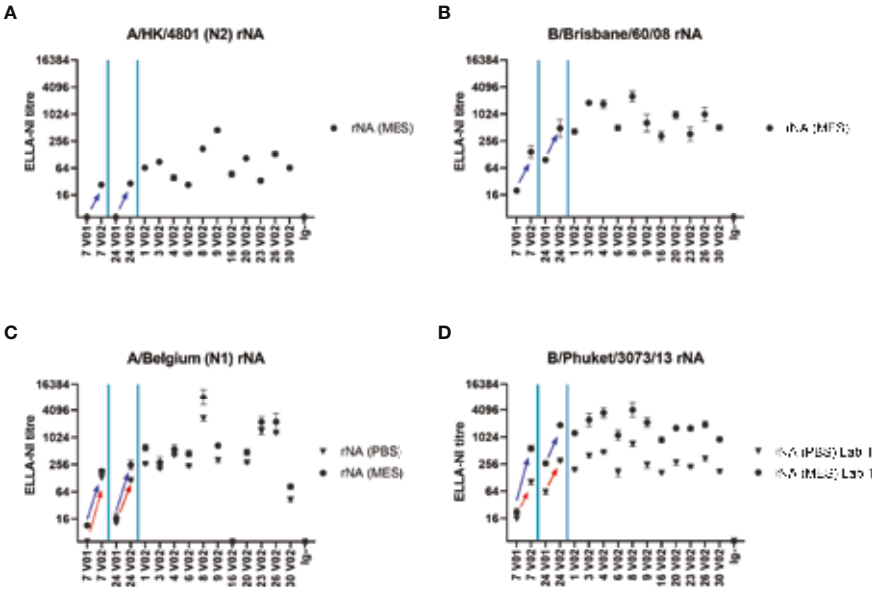

FIGURE 7 | Recombinant NA (rNA) antigen in ELLA-NI. A panel of 16 human sera were titrated in ELLA-NI using rNA from **(A)** A/Hong Kong/4801/2014 (N2) **(B)** B/Brisbane/60/2008 **(C)** A/Belgium/145-MA/2009 (N1) and **(D)** B/Phuket/3073/2013 using either MES (circles, all four rNAs tested) or PBS (triangles, A/Belgium and B/Phuket only) based-buffers. Paired pre- (V1) and post- (V2) vaccination sera from two individuals (7 and 24) were included in the serum panel. Increases in ELLA-NI titres after vaccination are indicated by arrows (blue for MES buffer and red for PBS buffer). GMT of 2-4 independent titrations is plotted with geometric SD error bars.

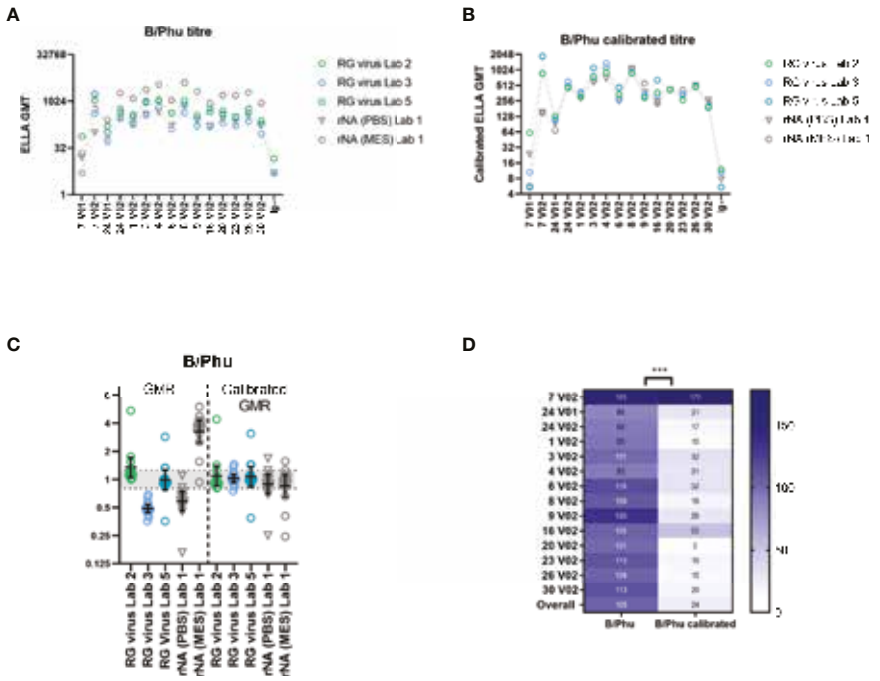

FIGURE 8 | Comparison of B/Phuket/3073/2013 rNA and mismatched HA/NA reverse genetics (RG) viruses in ELLA-NI and calibration using a mid-titre serum sample. **(A)** ELLA-NI titres for B/Phuket/3073/2013 (B/Phu) RG virus tested in three labs (Labs 2/3/5) and rNA from B/Phuket/3073/2013 tested in one lab with either a DPBS buffer [rNA (PBS)] or an MES buffer [rNA (MES)]. **(B)** ELLA titres as in [A] after calibration with mid-titre serum sample 20 V2. **(C)** Geometric mean ratios (GMR) of B/Phuket/3073/2013 RG virus and rNA ELLA-NI titres before and after calibration. The geometric mean and 95% CI are shown in black **(D)** %GCV per sample across the 5 labs/methods before and after calibration. *** indicates significance using the Wilcoxon matched pairs test [P=0.0002].

GMTs of rNA B/Phuket/3073/2013 tested in laboratory 1 using PBS buffer and MES buffer. All testing laboratories and methods give the same trend for all samples tested, however rNA NI titres when using MES buffer were substantially higher than those obtained using PBS buffer or using RG viruses.

Following the same approach applied to our previous data sets, calibration using the mid-titre sample 20 V2 improved inter-laboratory agreement. **Figure 8B** shows the GMTs for each sample tested with B/Phuket antigens after calibration, with closer agreement between different testing laboratories and antigens. **Figure 8C** shows the GMRs across all samples tested before and after calibration, with GMR becoming much closer to 1 (particularly evident for rNA testing using MES buffer), with the majority of GMR values falling within the indicative range of [0.8-1.25] (37% of GMR values fall within this range before calibration, rising to 77% after calibration). **Figure 8D** shows the %GCV before and after calibration, again showing a significant improvement in agreement between laboratories.

The same approach was also applied to ELLA-NI titres obtained using A/Belgium/145-MA/2009 (A/California/07/2009-like) rNA and ELLA-NI titres obtained using A/California/07/2009 NA mismatched RG virus (see **Supplementary Figure S5**). Calibration using mid-titre sample 20-V2 improved inter-laboratory agreement, with GMRs closer to 1 and %GCV decreased for the majority of samples.

These data show that tetrabrachion rNA could be used as an antigen in ELLA-NI testing as an alternative to RG influenza viruses; however when testing with MES buffer, ELLA titres could be substantially higher than observed when testing with RG viruses and show strain to strain variability. We show that a calibrator could be used to address this problem, highlighting the importance of developing standards for seasonal influenza serology.

DISCUSSION

FLUCOP aims to provide a toolbox of serological assays for influenza through two mains methods i) encouraging the use of consensus, harmonized SOPs that have been assessed in international collaborative studies, and ii) investigating the applicability and relevance of potential serology standards. To improve harmonization and inter-laboratory agreement of serological testing (essential for assays used to define correlates of protection), these assays need to be precise, robust, and in the case of influenza must also have minimal strain-strain variability in performance whilst additionally being able to differentiate between subtypes. As interest in NA as a target antigen for novel vaccines grows, standardized methods for testing functional anti-NA antibodies would greatly facilitate development and regulation of these novel vaccines. ELLA-NI has already been used for studying anti-NA antibody responses post vaccination (32) and in clinical trials (29). Building upon previous studies optimising ELLA-NI (19, 20) and studies showing ELLA-NI to be robust within a single laboratory setting (19, 20, 33), here we provide a detailed harmonized SOP (see **Supplementary Material**) demonstrated through our international collaborative studies to be precise, linear and robust when testing using N1 antigen.

Two essential attributes for influenza serology assays are strain-strain consistency in performance and ability to differentiate between influenza subtypes. Previous studies using ELLA-NI to detect antibodies to B influenza viruses have used WT viral antigens, where contribution of anti-HA antibodies to B virus NI titres could not be excluded (19). In this study we tested the specificity of two mismatched RG viruses with the NA from B Victoria and B Yamagata lineage viruses and the HA from an A/H9 virus for the first time. A previous study developed an H6 RG B Yamagata NA containing virus using a very similar approach to that used here (28) and demonstrated the virus performed well in ELLA studies. These data support the use of ELLA-NI for detection of both A and B influenza NA antibodies with consistent assay performance, and importantly differentiation between B Victoria and B Yamagata lineages with almost no cross reactivity. It would be interesting to test these mismatched RG B viruses with sera from previous or subsequent vaccination campaign years to assess how antigenic drift is captured by ELLA-NI. A previous study tested H1N1/H3N2 NA drift using the TBA assay (34) and H3N2/H2N2 NA antigens in ELLA-NI spanning 5 decades, demonstrating that drifted variants can be detected with ELLA-NI (20), but this remains to be shown for both N1 (in ELLA-NI) and B antigens.

Inter-laboratory agreement can be improved both by using harmonized protocols and by using a biological standard, allowing for the normalization and direct comparison of assay results regardless of the protocol used to derive them. Seasonal influenza presents a particular problem in regard to biological standards, as levels of antigenic drift are high (35). Different strains within a subtype will change antigenically over time, making definition of international units against individual strains difficult and impacting on the useable lifespan of a seasonal influenza biological standard. Nevertheless, standards have been proven to reduce inter-laboratory variation using HAI, Virus Neutralisation (VN) and ELLA-NI (21, 36–40) and warrant further development. In the absence of commercially available biological standards, we selected a mid-titre human serum sample from each panel tested as a calibrator. One previous study has looked at the inter-laboratory performance of ELLA using less strict harmonization criteria for testing than used in this study (21). An overall %GCV of 112% was calculated for N1 antigen (falling to 59% after normalization with a standard). Our use of a detailed harmonized SOP along with harmonized data analysis appears to give lower variation than previously observed (overall %GCV for our precision data of 78% falling to 20% after calibration with a standard), however it should be noted that a greater number of labs participated in that previous study, leading perhaps to increased variability. Acceptance criteria for background levels and titration of antigen were identified as critical for improved inter-laboratory agreement (21) – both these considerations have been included in the harmonized SOP presented here. Our use of a calibrator clearly and significantly reduced inter-laboratory variation in every collaborative study carried out, demonstrating the consistent improvement across studies using multiple influenza strains. The continued development of seasonal standards for

influenza remains a priority. Work is also required to investigate the best source of a biological standard. Pools of human sera were used in this study, where large volumes can be created through pooling of multiple donors. Other potential sources include antisera from animals – this approach has the added advantage of inoculating animals with recombinant NA to avoid any interference of anti-HA antibodies. However the ethical burden of this approach must be taken into consideration. It will be important to investigate the possible lifespan of a standard. The fact that NA experiences slower antigenic drift than HA (9) may be advantageous and prolong the lifespan of a seasonal standard for NA.

One drawback to using ELLA is the interference of anti-HA antibodies in the assay. In this study we initially confirmed the specific role of HA antibodies in generating false positive titres, or in overestimating NI titres. This is in agreement with other studies using both ELLA and the TBAA (19, 24, 41). Kosik et al. show that HA-specific mAbs can inhibit NA activity only when HA is in close proximity to NA (an effect abrogated by detergent disruption of virions) (24), and they suggest two mechanisms through which HA antibodies interfere with ELLA: firstly, that HA binding to the fetuin glycoprotein facilitates NA activity (and blocking this binding reduces NA activity) and, secondly, that HA antibodies can sterically hinder NA activity by blocking the active site of the enzyme (24). These data taken together clearly demonstrate the need to use mismatched antigens containing HA not circulating in the human population, or an alternative source of antigen such as rNA or lentiviral pseudotypes.

rNA has previously been tested as an antigen in ELLA for a limited number of influenza strains [N1 from H5N1 A/Vietnam/1203/2004 (23), N1 from A/California/07/2009 and A/turkey/Turkey/01/2005 (42)]. rNA has several advantages over live virus as a source of NA for ELLA; rNA is safer to produce and handle than RG mismatch viruses without the need for high containment level facilities; rNA is easier to produce for non-influenza specialist laboratories [for example expression of tetrabrachion rNA in mammalian cells (42)] and is commercially available for some influenza strains, although this remains an expensive source of rNA.

In this study we successfully used tetrabrachion rNA against all four seasonal influenza vaccine components. Some interesting differences are observed in NI titres when testing with two different buffers, with an acidic MES buffer giving higher titres (substantially so for B/Phuket NA) than PBS buffer. MES buffer was initially selected as it has been reported that some influenza strains have impaired NA activity at neutral pH (18). B/Phuket NA appears to be pH sensitive, with higher NI titres in MES-based acidic buffer, in contrast to A/Belgium NA which does not appear to be so pH sensitive. It is not clear however, if a difference in NI titre is due to pH or perhaps the concentration of calcium within the buffer; calcium concentration is higher in MES buffer than PBS, and calcium binding is known to be important in NA activity (43–45) and NA thermostability (42, 45, 46).

Despite the differences in titre observed when using different buffers and different NA sources, the use of a calibrator reduced variation and results were comparable, with overall GCV falling from 105% to 24%. These results demonstrate that whilst rNA can be used as a source of NA in ELLA care needs to be taken in the absence of commercially available standards when comparing rNA and RG mismatched virus ELLA-NI titres.

Alternative sources of NA for ELLA-NI not investigated in this study would be detergent disrupted WT virus, or lentiviral pseudotypes expressing the NA of interest with a mismatched HA, or without HA. Lentiviral pseudotypes have been shown to give comparable titres for RG mismatched viruses for A/California/07/2009 N1 and A/Hong Kong/4801/2014 N2 (26), and it would be interesting to further test these as a source of NA using our harmonized protocol in future work.

In summary, in this study we have provided a detailed harmonized SOP for ELLA-NI, we have validated this SOP in a multi-laboratory collaborative study showing the assay had consistent precision, linearity and robustness using an N1 antigen and had influenza type specificity, including differentiation between B Yamagata and B Victoria lineages. We have shown that ELLA-NI gave consistent results when testing A and B influenza RG mismatched viruses, and additionally that rNA could be used as an alternative source of NA in the assay. We show that, in the absence of a commercially available standard, a calibrator significantly improved both inter-laboratory agreement and agreement in testing between RG mismatched viruses and rNA sources. Our results support the further development of seasonal influenza serology standards. Altogether, the validated ELLA-NI procedure and the additional specific aspects investigated are considered of great value for the harmonized and sound evaluation of immunogenicity of novel influenza vaccines and could be readily included into existing regulatory recommendations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Universitair Zeikenhuis Gent, Commissie vor medische ethiek (committee for medical ethics) Belgian registration number B670201733136, and the Human Biological Sample Operating Committee (HBSOC) Sanofi Pasteur. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by UK Home Office Licences and approved by NIBSC's Animal Welfare and Ethics Review Body (AWERB). BALB/c ByJ mice were housed at Sanofi Pasteur, SA. The pooled sera were prepared from an Animal Use Protocol reviewed by the Ethics Committee #11 of Sanofi Pasteur. All experiments were conducted in accordance with the European Directive 2010/63/UE as published in the French Official Journal of February 7th, 2013.

AUTHOR CONTRIBUTIONS

Conceptualization and study design M-CB, HS-S, TO, DF, SG, KH, RJC, RW, EM, JPW, JW, and OE. Laboratory work HS-S, SC, LS, JW, FZ, SH, CMT, SM, SW, PA, and VL. Data analysis DF, SG, TO, and JW. Writing and Editing JW, M-CB, HS-S, SG, TO, CMT, SM, OE, and RW. All authors had full access to the data and approved the final draft of the manuscript before it was submitted by the corresponding author.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.909297/full#supplementary-material

Supplementary Figure 1 | Structure of chimeric RG influenza B viruses. The chimeric viruses containing HA of H9 and NAs from influenza B viruses were generated by reverse genetics technique using the pHW2000 plasmid as described earlier (30). The HA of these strains is a chimeric protein consisting of HA ectodomain of the H9N2 strain A/chicken/Beijing/2/97, and CT+TM (cytoplasmic tail + transmembrane region) from seasonal H1N1 strain A/Brisbane/59/2007. The NA of these viruses are also chimeric proteins containing an ectodomain (stalk and head) of the influenza B viruses (B/Brisbane/60/2008 or B/Phuket/3073/2013) and CT+TM from seasonal H1N1 strain A/Brisbane/59/2007.

Supplementary Figure 2 | Intra-laboratory variation is consistent when testing multiple influenza subtypes. Three mismatched influenza viruses were tested using the FLUCOP ELLA-NI SOP. A serum panel of 16 samples was tested in three laboratories. Each laboratory carried out three independent runs. %GCV per sample is shown for Lab 2 (Green), Lab 3 (Blue) and Lab 5 (Teal) for each virus: B Bris (H9 with B/Brisbane/60/2008 NA, circles), B Phu (H9 with B/Phuket/3073/2013 NA, squares) and A Cal (H7N1 with A/California/07/2009 NA, triangles). Negative samples 12 and Ig- were excluded from analysis. %GCV is uniform across laboratory, sample and virus strain.

Supplementary Figure 3 | Specificity of the ELLA-NI using monospecific ferret sera. Three mismatched influenza strains containing the neuraminidase from B/Phuket/3073/2013 (B/Phuket NA), B/Brisbane/60/2008 (B/Bris NA) and A/California/07/2009 (A/Cal HA) were tested with sera from ferrets challenged with a single strain of influenza (two individual ferrets per strain): B/Phuket/3073/2013 (B/Phuket Ferret 1 and 2); B/Brisbane/60/2008 (B/Bris Ferret 1 and 2) and A/California07/2009 (A/Cal Ferret 1 and 2). GMTs of at least two independent replicates are shown.

Supplementary Figure 4 | Inter-laboratory performance before and after calibration. Three strains of influenza **(A)** H9 with B/Brisbane/60/08 NA, **(B)** H9 with B/Phuket/3073/13 NA and **(C)** H7N1 with A/California/07/09 NA, were tested using the FLUCOP ELLA-NI SOP. ELLA-NI titres are shown before (black) and after (blue) calibration using serum sample 20V02. Two pre-(V01)/post-(V02) vaccinations pairs were included in the testing (7V01/02 shaded blue and 24V01/02 shaded in green).

Supplementary Figure 5 | Comparison of A/Belgium/145-MA/2009 rNA and mismatched HA/NA reverse genetics (RG) virus containing A/California/07/2009 NA in ELLA-NI and calibration using the mid-titre serum sample 20 V2. **(A)** ELLA-NI titres for A/California/07/2009 RG virus tested in three labs (A/Cal RG virus Lab 1/2/3) and rNA from A/Belgium/145-MA/2009 tested in one lab with either a DPBS buffer (A/Belgium rNA (PBS)) or an MES buffer (A/Belgium rNA (MES)). **(B)** ELLA-NI titres as in [A] after calibration with mid-titre serum sample 20 V2. **(C)** GMR of A/California/07/2009 RG virus and A/Belgium/145-MA/2009 rNA ELLA-NI titres before and after calibration. **(D)** %GCV per sample across the 5 labs/methods before and after calibration. * indicates significance using the Wilcoxon matched pairs test [P=0.0295].

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Efficacy and safety of a quadrivalent influenza vaccine in children aged 6–35 months: A global, multiseasonal, controlled, randomized Phase III study

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ABSTRACT

Background: Children are an important target group for influenza vaccination, but few studies have prospectively evaluated influenza vaccine efficacy (VE) in children under 3 years of age. This was a randomized Phase III trial to assess the efficacy, immunogenicity, and safety of an inactivated quadrivalent influenza vaccine (QIV) in young children (EudraCT: 2016–004904–74).

Methods: Influenza-naïve children aged 6–35 months were randomized during three influenza seasons to receive vaccination with QIV or a non-influenza control vaccine. One group of participants was revaccinated with QIV in the subsequent influenza season. The primary efficacy endpoint was the absolute VE of QIV against influenza caused by any circulating strain. Key secondary efficacy endpoints included the absolute VE of QIV against influenza due to antigenically matching strains and immunogenicity. Safety and reactogenicity were also evaluated.

Results: In total, 1005 children received QIV and 995 received control vaccine. Influenza A/B infection due to any circulating influenza strain occurred less frequently in children who received QIV *versus* children receiving a control vaccine. The absolute VE of QIV against any circulating influenza strain was 54% (95% confidence interval [CI]: 37%, 66%). The absolute VE of QIV against antigenically matching influenza strains was 68% (95% CI: 45%, 81%). Mean hemagglutination inhibition titers for all influenza strains in the QIV group increased post-vaccination, whereas increases were minimal in the control vaccine group; results from virus neutralization and neuraminidase-inhibition assays were generally consistent with the hemagglutination inhibition assay findings. Approximately 12 months after primary vaccination with QIV, antibody titers remained higher than pre-vaccination titers for most strains. In participants who were revaccinated, QIV elicited strong antibody responses. The overall safety profile and reactogenicity of QIV was comparable with control vaccine.

Conclusion: Primary vaccination with QIV was well tolerated and effective in protecting children aged 6–35 months against influenza.

1. Introduction

Influenza A and B viruses are respiratory pathogens that occur globally and cause seasonal influenza epidemics as well as sporadic out of season outbreaks [1]. Influenza is associated with significant morbidity and mortality worldwide; the World Health Organization (WHO) estimates that influenza epidemics result in ~ 1 billion infections, 3–5 million cases of severe illness, and 300 000–500 000 deaths every year [2]. Symptoms of influenza can range from a mild respiratory disease confined to the upper respiratory tract

Abbreviations: AE, adverse events; AESI, AE of special interest; CI, confidence interval; CV, control vaccine; E, number of events; HI, hemagglutination inhibition; HR, hazard ratio; MAE, medically attended AE; NI, neuraminidase inhibition; NCI, new chronic illnesses; NH, northern hemisphere; QIV, quadrivalent influenza vaccine; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; SAE, serious AEs; SH, southern hemisphere; TC, telephone call; TEAE, treatment-emergent AE; TIV, trivalent influenza vaccine; VE, vaccine efficacy; VN, virus neutralization; WHO, World Health Organization.

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to severe and potentially fatal pneumonia, and infection can lead to a number of non-respiratory complications that affect the heart, central nervous system, and other organ systems [2].

Since 1968, influenza A(H3N2) strains have circulated in the human population and are currently the major cause of seasonal influenza morbidity and mortality followed by influenza B strains and A(H1N1) strains [3,4]. Influenza B viruses split into two antigenically distinct lineages (B/Yamagata and B/Victoria) in the early 1980s and these lineages now cocirculate worldwide [5]. While seasonal influenza outbreaks occur every year, influenza pandemics are far rarer and more threatening due to their unfamiliarity and potential for catastrophic impact [6]. Several influenza pandemics have occurred in the past century; most recently, the A(H1N1) pandemic that originated in Mexico in 2009 was linked to >18 000 deaths globally, although the true mortality rate is generally accepted to be far higher [6,7].

Vaccination is recognized as a fundamental component of influenza prevention and control [6]. Children, pregnant women, and elderly people are at particular risk of complications following influenza infection [8–13]. Consequently, the WHO recommends annual vaccination for the following most vulnerable population groups: pregnant women, children aged 6–59 months, the elderly, people with specific chronic medical conditions, and healthcare workers [1].

Traditional influenza vaccination strategies previously used a trivalent vaccine that contains two influenza A strains (H1N1 and H3N2) and one B strain. As trivalent influenza vaccines (TIVs) only include one selected B lineage, they may provide limited immunity against strains of the other B lineage; consequently, TIVs may have reduced effectiveness against influenza B epidemics during seasons in which a significant proportion of the disease is caused by opposite lineage influenza B strains [14]. Quadrivalent influenza vaccines (QIVs) seek to circumvent this problem by incorporating both Yamagata and Victoria B lineages, in addition to the influenza A strains (H1N1 and H3N2), in order to offer broader protection [14].

Antigenic drift can result in a mismatch between the strains contained in the vaccine and the predominant circulating strains, which results in variable levels of vaccine effectiveness from year to year. Consequently, vaccination is recommended annually to ensure optimal match between the vaccine and the prevailing influenza strains and because influenza vaccines can provide a relatively short duration of protection of around 6 months [1,15].

Children are an important target group for influenza vaccination. The annual incidence rate for influenza in children is estimated at 20–30% (compared with 5–10% in adults) and worldwide each year, influenza leads to the hospitalization of around 870 000 children under 5 years of age [1,16]. Influenza B is a particular concern in young children in whom it may cause a disproportionate burden of severe illness and hospitalization [17,18], thus underscoring the importance of adding another B strain to influenza vaccines for the protection of pediatric populations. Annual vaccination against influenza is expected to protect children from an increased risk of complications and severe illness [8,10]. In addition, children are thought to be important transmitters of influenza in the community because they are in close contact with each other in schools and childcare settings, have close contact with adults and the elderly, often have poor hygiene habits and limited pre-existing immunity, and shed high virus titers [1,16]. Evidence suggests that vaccination of children may help reduce viral transmission by conferring protection not only to the child, but also indirect protection to unvaccinated household members and community contacts [1,19,20].

Previous studies have shown QIVs to elicit immunogenicity and efficacy in children aged 3–17 years and adults [21–27] but, despite the WHO recommendations to vaccinate children from as young as 6 months [1], only a limited number of studies have evaluated vaccine efficacy in the prevention of influenza in children younger than 3 years of age [28,29]. This randomized Phase III study aimed to assess the efficacy, immunogenicity, and safety of an inactivated (surface antigen) QIV in influenza vaccine and illness-naïve children aged 6–35 months in comparison with non-influenza (child) control vaccines.

2. Materials and methods

2.1. Study design

This was a Phase III, randomized, observer-blind, comparator-controlled study to assess the efficacy and safety of an inactivated (surface antigen) QIV in children aged 6–35 months. The trial was conducted at 56 centers across Europe and Asia between 1 September 2017 (first subject, first visit) and 31 January 2020 (last subject, last visit).

The study was conducted across three influenza seasons, which included two NH seasons (2017/2018 and 2018/2019) and one SH season (2019) (Fig. 1). Study participants who received vaccination with QIV in Year 1 (NH 2017/2018 season) were revaccinated with QIV in Year 2 (NH 2018/2019 season) to assess immunogenicity following revaccination. All other study participants were vaccinated only in the NH 2018/2019 or SH 2019 season. The revaccination section of the study was open-label and was designed to assess immunogenicity; it was not intended to evaluate efficacy.

Participants vaccinated in the NH 2017/2018 season were followed until 15–31 May 2018; those vaccinated or revaccinated in the NH 2018/2019 season were followed until 15–31 May 2019; and those vaccinated in the SH 2019 season were followed until 15–31 January 2020.

The study was approved by local institutional review boards and complied with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from at least one parent or legally acceptable representative of each child (in accordance with the applicable local regulations and guidelines) before enrollment. The trial was registered with the European Union Clinical Trials Register (EudraCT number 2016–004904–74).

2.2. Study participants

Eligibility screening was performed at Visit 1 or a maximum of 2 weeks before Visit 1. Eligible participants were males and females and aged between 6 and 35 months inclusive on the day of receiving the first study vaccination. Participants were enrolled at pediatric sites or vaccination centers if they were judged by the investigator to be in stable health. Individuals with underlying chronic disorders were eligible for inclusion if their symptoms or signs were controlled and if the underlying condition was not the reason for influenza vaccination. Medication for pre-existing conditions was permitted if the dose was stable for at least 3 months before enrollment. Participants aged between 6 and 24 months on Visit 1 were required to have been born at full term ($\geq$37 weeks of gestation) with a birth weight of $\geq$2.5 kg.

Participants were not eligible for enrollment if they had ever been vaccinated against influenza, had ever been diagnosed with laboratory-confirmed influenza, had received any vaccine (including routine childhood vaccines) within the previous 28 days, or were due to undergo any vaccinations within 28 days following the planned study vaccinations. Other key exclusion criteria included history of serious adverse reaction to any vaccine; history of Guillain-Barré syndrome; administration of immunosuppressants for >14 days within the previous 3 months or planned use

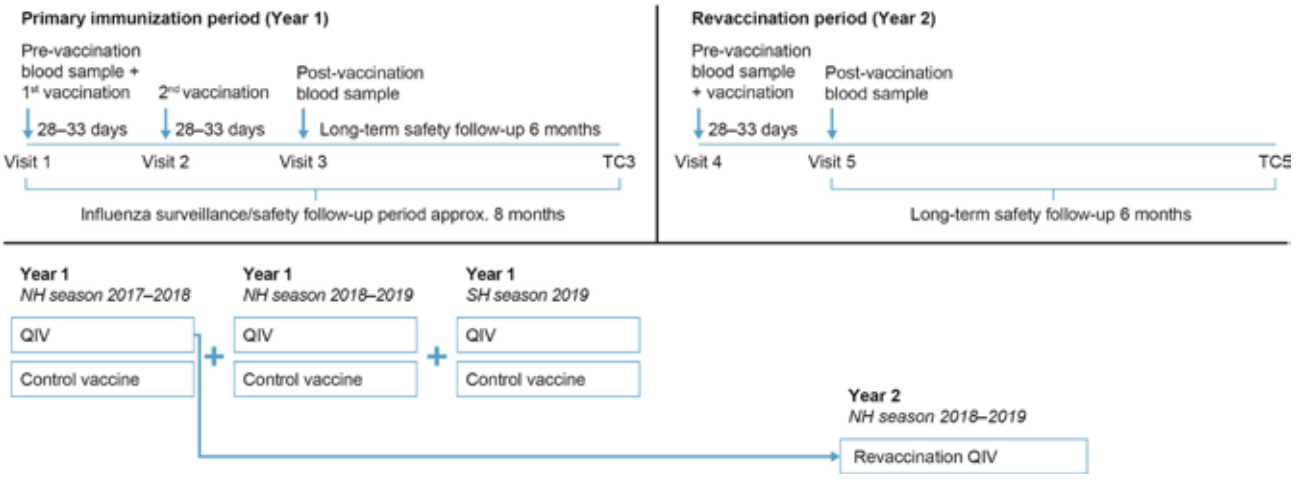

Fig. 1. Study design. NH, northern hemisphere; QIV, quadrivalent influenza vaccine; SH, southern hemisphere; TC, telephone call.

during the study (topical use of corticosteroids was permitted); administration of immunoglobulins and/or any blood products within the previous 3 months or planned use during the study; administration of cytotoxic drugs, anticancer chemotherapy, or radiation therapy; ongoing aspirin therapy; any confirmed or suspected immunosuppressive or immunodeficient condition; and history of solid organ or bone marrow/stem cell transplant.

2.3. Study procedures

Participants were stratified into the following age groups: 6–11 months, 12–18 months, 19–24 months, and 25–35 months. Participants were then randomized (1:1) to receive two doses of either QIV (0.5 mL per dose; Influvac® Tetra, Abbott) or a non-influenza control vaccine (0.25–0.5 mL per dose); both were given intramuscularly approximately 28–33 days apart (Fig. 1). Participants who were revaccinated with QIV in Year 2 (NH 2018/2019 season) received one dose of QIV in the second season.

The QIV contained 15 μg hemagglutinin of each of the viral strains recommended by WHO for the NH 2017/2018 season, NH 2018/2019 season, or SH 2019 season. Briefly, there was no change in the A(H1N1) and B/Yamagata strains across the three seasons, the A(H3N2) strain was different for every season, and the B/Victoria strain was consistent in the NH 2018/2019 and SH 2019 seasons but was different for the NH 2017/2018 season. Additional vaccine characteristics are summarized in **Supplementary Table S1.**

The control vaccine administered depended on age. Subjects aged 6–11 months received either pneumococcal conjugate vaccine (Prevenar 13®; Pfizer) or meningococcal group C conjugate vaccine (Menjugate®; GlaxoSmithKline); those aged 12–35 months received either hepatitis A vaccine (Havrix Junior®; GlaxoSmithKline), tick-borne encephalitis vaccine (TicoVac Junior®/FSME-IMMUN Junior®; Pfizer), or varicella vaccine (Varivax®/Provarivax®; Merck, Sharp & Dohme). Additional vaccine characteristics are summarized in **Supplementary Table S1.**

Participants were randomized using a centralized electronic system (interactive voice/computer/remote response system). Packaged medication was provided to the study sites. Data were collected in an observer-blinded manner whereby participants, parents, investigators, and parties responsible for study endpoint evaluations and review/analysis of study data were unaware of treatment assignments. To maintain blinding vaccinations were performed using masking by authorized study personnel who did not participate in the study's clinical evaluations and serological results were not available during the study to anyone involved in the clinical conduct of the study. In addition, the control vaccines followed the same study vaccine administration schedule.

Participants were tested for influenza-like illness if the following case definition was met (in line with the European Centre for Disease Prevention and Control definition [30]): sudden onset of symptoms; at least one of the following four systemic symptoms: fever, malaise, headache, or myalgia; and at least one of the following three respiratory symptoms: cough, sore throat, or shortness of breath. Fever was defined as a temperature of ≥38.0 °C if measured rectally or ≥37.5 °C if measured axillary (if rectal method was not possible).

2.4. Study objectives

The primary efficacy objective was to demonstrate the absolute efficacy of the QIV (efficacy *vs* non-influenza control vaccine) in the prevention of symptomatic influenza infection due to any circulating seasonal influenza strain. Vaccine efficacy (VE) was defined as the fraction of influenza cases directly prevented by vaccination.

Key secondary efficacy objectives were to demonstrate the absolute efficacy of the QIV in the prevention of symptomatic influenza infection of antigenically matching influenza strains; to describe the immunogenicity of each of the strains in the QIV with respect to hemagglutination inhibition (HI) in all participants and virus neutralization (VN) and neuraminidase inhibition (NI) in a random subset of participants from those vaccinated in NH 2017/2018 season and revaccinated in NH 2018/2019 season; and to describe Year 2 baseline and post-vaccination immunogenicity for each of the strains in the QIV with respect to HI, as well as VN and NI, in children exposed to the QIV in Year 1 who were revaccinated in Year 2.

The safety objective was to evaluate the safety and reactogenicity of the study vaccines.

2.5. Study endpoints and analyses

The primary efficacy endpoint was the first occurrence of reverse transcription-polymerase chain reaction (RT-PCR)-confirmed influenza A and/or B illness of any severity caused by any circulating seasonal influenza strain occurring between 28 days following the second vaccine administration and the end of the influenza surveillance period. Absolute VE (expressed as a percentage) was estimated as 1 minus the relative risk of influenza infection for QIV-vaccinated subjects.

Key secondary efficacy endpoints were the first occurrence of confirmed influenza A and/or B illness of any severity due to antigenically matching influenza strains occurring between 28 days following the second vaccine administration and the end of the influenza surveillance period; and the immunogenicity of each strain in the QIV, which was determined by assessing the post-vaccination geometric mean antibody titers for HI (in all participants) and VN/NI (in the subset of participants who were revaccinated in NH 2018/2019 season) and their change from baseline. Seroconversion rates and geometric mean fold increases for the vaccine strains were also assessed.

Blood samples for determination of the primary immune response were collected at Visit 1, immediately prior to the first dose of vaccine (pre-vaccination), and at Visit 3, 28–33 days after the second dose of vaccine (post-vaccination) (Fig. 1). In participants who were revaccinated with QIV in Year 2, blood samples for determination of immunogenicity in the reimmunization period were collected at Visit 4, immediately prior to revaccination, and at Visit 5, 28–33 days post-vaccination, thus assessing immunogenicity from the previous season as well as the subsequent one (Fig. 1).

The occurrence of solicited (prespecified in the subject diary) injection-site adverse events (AEs) and systemic adverse reactions were assessed within 7 days following each vaccination. At Visits 1, 2, and 4, parents or legal representatives were provided with a diary, thermometer, and ruler for daily reporting of solicited local and systemic reactions and overall inconvenience occurring during the first 7 days after each vaccination. Occurrence of any unsolicited (spontaneously reported) AEs was assessed for up to 28–33 days following each study vaccination. Each participant was evaluated for any AEs from Visit 1 until Visit 3; revaccinated subjects were also evaluated from Visit 4 until Visit 5. Serious AEs (SAEs), new chronic illnesses (NCIs), AEs of special interest (AESIs), and medically attended AEs (MAEs) were also assessed for the full safety follow-up periods (6–8 months).

2.6. Sample size calculation

The average influenza attack rate was assumed to be ≥4.5% among unvaccinated subjects along with a VE of ≥60%, corresponding to an attack rate of ≤1.8% among vaccinated subjects. With these assumptions, 2000 participants would be needed to ensure a statistical power of ≥90% using a two-sided significance level of 5%. Allowing for a 10% failure rate, the planned number of participants to be screened was 2200.

2.7. Statistical analyses

The full analysis set included all subjects who were in the safety set and provided primary efficacy data; the full analysis set was used for determination of VE. The safety set included all participants who were randomized, vaccinated, and provided at least one post-vaccination safety observation.

Relative risk was estimated by the hazard ratio (HR) using the Cox proportional hazards model. The VE was derived as VE = 1–HR. Age group (6–11, 12–18, 19–24, and 25–35 months), season, and country were included in the model as indicator variates; the reported VE estimates are therefore adjusted estimates.

Immunogenicity data were analyzed for the primary immunization and revaccination immunogenicity samples. Summary statistics calculated for each of the strains included reverse cumulative distribution curves for HI (in all participants) and VN/NI (in the subset of participants who were revaccinated in NH 2018/2019 season); pre-first vaccination and post-second vaccination geometric mean titers and geometric mean fold increase for HI, VN, and NI; and seroconversion rates for HI, VN, and NI, defined as becoming seropositive if seronegative at enrollment, or a ≥4-fold rise in titer if seropositive (i.e. detectable titer) at enrollment.

3. Results

3.1. Subject disposition and baseline demographics

In total, 2007 subjects were randomized. Of these, 2000 were vaccinated (QIV, n = 1005; control vaccine, n = 995), 1961 (97.7%) completed the primary immunization period, and 334 subjects were revaccinated in Year 2 (**Supplementary** Fig. 1). All 2000 vaccinated participants were included in the safety and full analysis sets. The primary immunogenicity and revaccination immunogenicity samples included 1842 (91.8%) and 294 (88.0%) subjects, respectively. Overall, there were 46 (2.3%) premature discontinuations during the primary immunization period due to withdrawal of consent (QIV, n = 14; control vaccine, n = 9); protocol violations (QIV, n = 2; control vaccine, n = 2); AEs (QIV, n = 1; control vaccine, n = 1); and administrative reasons (QIV, n = 1; control vaccine, n = 1). Fifteen subjects were lost to follow-up (QIV, n = 5; control vaccine, n = 10).

Baseline subject demographics are shown in Table 1. In general, the demographics of participants were similar across vaccination groups. In the age subgroups, the demographics of subjects were similar between the QIV and control groups and consistent with those of the overall population. Of the participants who were revaccinated at Year 2, the majority were white (98.5%) and 58.1% were female. The mean age of revaccinated subjects at the time of screening was 19.9 months, with the majority (55.4%) in the 19–35 months age group.

3.2. Absolute vaccine efficacy of the QIV in the prevention of symptomatic influenza infection due to any circulating influenza strain (primary efficacy objective)

The incidence of influenza A/B infection due to any circulating influenza strain was lower in the group of children receiving the QIV *versus* children receiving a control vaccine (Fig. 2**A**). HR for the VE of QIV was 0.46; therefore, VE expressed as a percentage was 54% (95% confidence interval [CI]: 37%, 66%), confirming the

Table 1
Baseline demographics for all randomized subjects and those who were revaccinated in Year 2 using second season strains. QIV, quadrivalent influenza vaccine; SD, standard deviation.

	Primary immunization		Revaccinated subjects
	QIV (n = 1009)	Control vaccine (n = 998)	QIV (n = 334)
Mean age (SD), months	19.4 (8.1)[a]	19.6 (8.3)[a]	19.9 (7.9)[a,b]
Age category, months	200 (19.8)[a]	195 (19.5)[a]	61 (18.3)[a,b]
6–11, n (%)	291 (28.8)[a]	281 (28.2)[a]	88 (26.3)[a,b]
12–18, n (%)	216 (21.4)[a]	217 (21.7)[a]	80 (24.0)[a,b]
19–24, n (%)	302 (29.9)[a]	305 (30.6)[a]	105 (31.4)[a,b]
25–35, n (%)			
Gender			
Male, n (%)	492 (48.8)	499 (50.0)	140 (41.9)
Female, n (%)	517 (51.2)	499 (50.0)	194 (58.1)
Race			
White, n (%)	751 (74.4)	733 (73.4)	329 (98.5)
Asian, n (%)	244 (24.2)	240 (24.0)	2 (0.6)
Black, n (%)	5 (0.5)	6 (0.6)	0
Other, n (%)	9 (0.9)	19 (1.9)	3 (0.9)

[a] Age was calculated relative to screening.
[b] Approximately 1 year prior to revaccination.

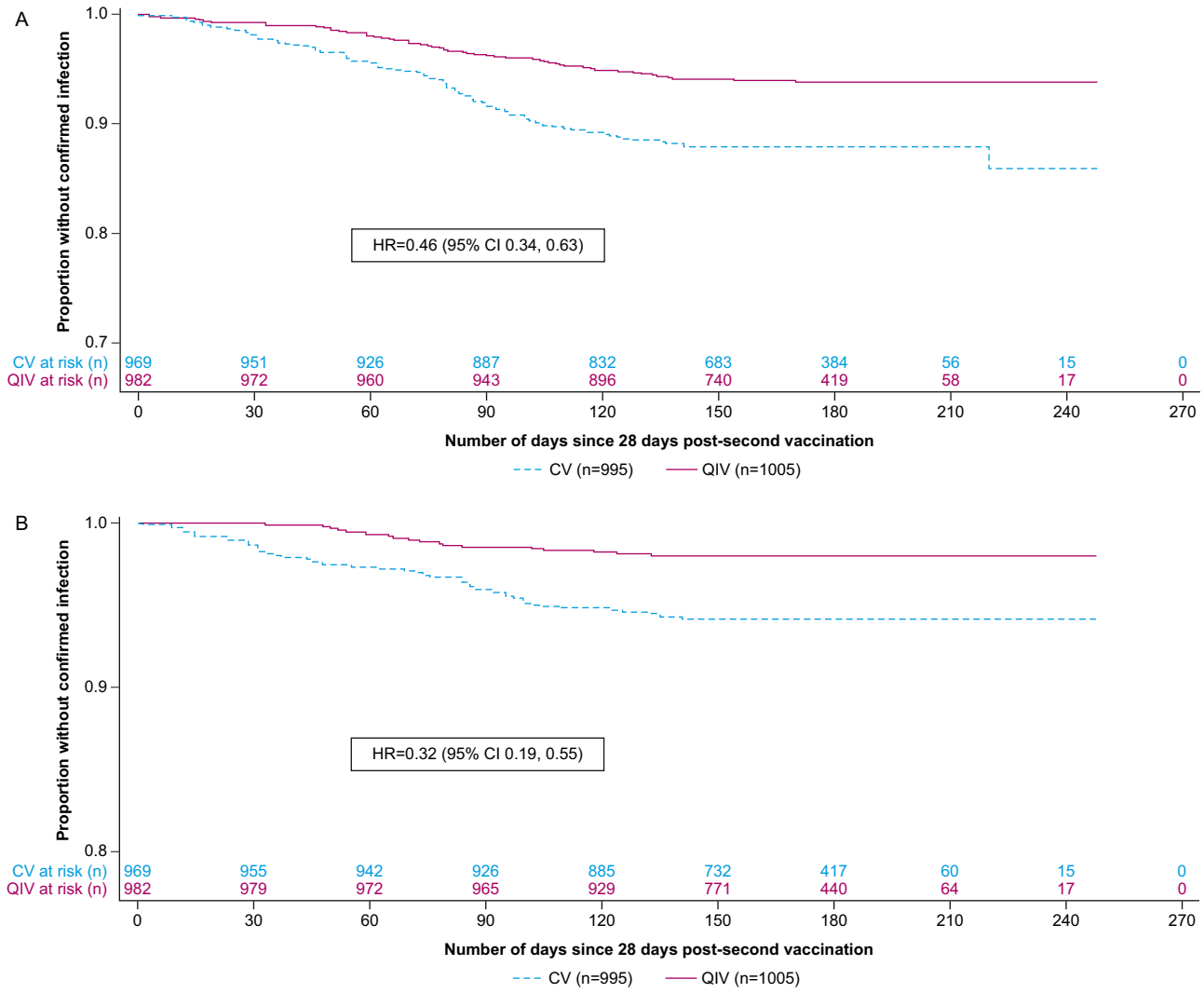

Fig. 2. Kaplan-Meier plot of time to first RT-PCR-confirmed influenza A and/or B infection in children aged 6–35 months due to A) any seasonal strain and B) antigenically matching influenza strains. CI, confidence interval; CV; control vaccine; HR, hazard ratio; QIV, quadrivalent influenza vaccine; RT-PCR, reverse transcription-polymerase chain reaction.

absolute VE of QIV in the prevention of symptomatic influenza due to any circulating strain. Between age groups, the efficacy of QIV ranged from 50% to 73%, except for the 6–11 months age group (21%) (**Supplementary** Fig. 2**A**).

3.3. Absolute vaccine efficacy of QIV in the prevention of symptomatic influenza infection due to antigenically matching influenza strains (key secondary efficacy objective)

The incidence of influenza A/B infection due to antigenically matching influenza strains was also lower in the QIV group *versus* the control group (Fig. 2**B**). The absolute VE against antigenically matching influenza strains was 68% (95% CI: 45%, 81%), confirming the absolute VE of QIV in the prevention of symptomatic influenza due to antigenically matching strains. The VE of QIV for antigenically matching influenza strains was consistent across age groups, ranging from 64% to 79% (**Supplementary** Fig. 2**B**). The VE of QIV among children aged 6–24 months (69%; 95% CI: 41%, 84%) was comparable with that in children aged 25–35 months (65%; 95% CI: 18%, 85%) (**Supplementary** Fig. 2**B**). VE was higher among subjects in the NH (combined seasons [2017/2018 and 2018/2019]; 73%; 95% CI: 50%, 85%) compared with those in the SH (season [2019]; 42%; 95% CI: −60%, 79%), and consistent with the results for the overall population.

Antigenic typing of influenza infections indicated a high degree of matched virus for the A(H1N1) strain and B lineage strains (Victoria and Yamagata), but a near complete mismatch for the A (H3N2) strains (**Supplementary Table S2**). VE estimates were generally comparable for RT-PCR-confirmed influenza A(H1N1) strain and B strain types, and higher than estimates for the A(H3N2) strains. (**Supplementary** Fig. 3**A**). VE due to antigenically matching strains was shown for the influenza B/Yamagata lineage strain and for the A(H1N1) strain, but not for the B/Victoria lineage strain (the CI crossed 0) (**Supplementary** Fig. 3**B**).

3.4. Immunogenicity

3.4.1. Primary immune response

Geometric mean HI titers increased from pre-vaccination to post-vaccination (Visit 3) for all strains in the QIV group (Fig. 3**A**) compared with minimal increases in the control group across all strains. **Supplementary Table S3** provides an overview of geometric mean fold increases and seroconversion rates for participants who received vaccination in NH 2017/2018 season. For partici-

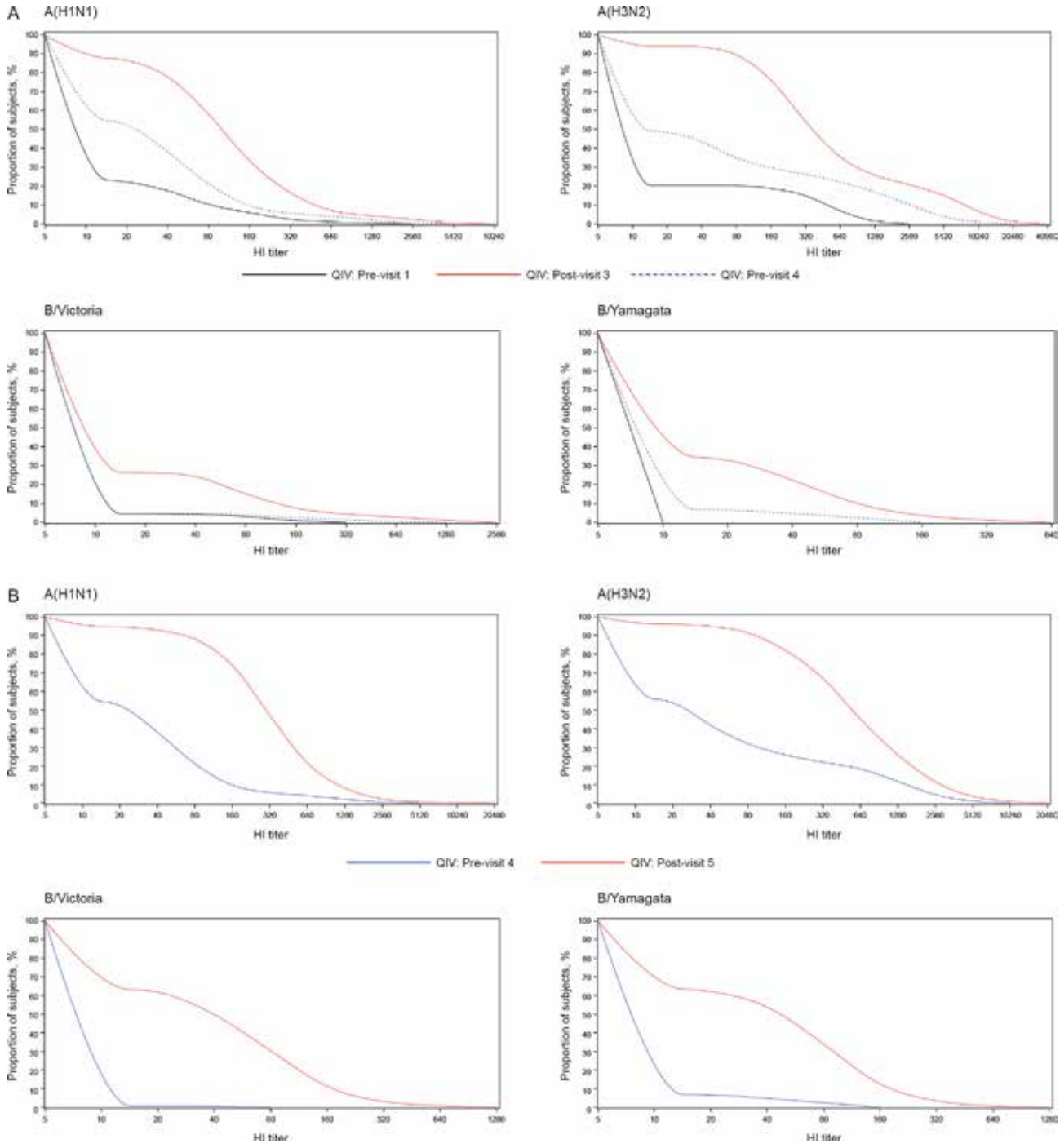

Fig. 3. HI titers for A) primary and long-term immune response and B) response following revaccination in subjects who received vaccination with QIV in NH 2017/2018 season and were revaccinated in Year 2 using second season strains. HI, hemagglutination inhibition; NH, northern hemisphere; QIV, quadrivalent influenza vaccine.

pants who received vaccination with QIV in NH 2017/2018 season and were revaccinated in Year 2, pre-vaccination geometric mean HI titers were broadly similar between the QIV and control groups for all strains. HI data from other seasons were generally consistent with those from the NH 2017/2018 season. Results of the VN and NI assays were generally consistent with results of the HI assay (**Supplementary** Fig. 4**A and** 5**A; Supplementary Table S3**).

3.4.2. Long-term immune response

Approximately 12 months after primary vaccination with QIV (Visit 4), geometric mean antibody HI titers remained moderately higher than pre-vaccination titers for both A strains and the B/Yamagata lineage strain, indicating the long-term persistence of the immune response in subjects who were due to be revaccinated (Fig. 3**A**). For the B/Victoria lineage strain, HI titers at Visit 4 had decreased to a level comparable with pre-vaccination titers. Persistence of the immune response for 12 months for the A(H3N2) strain and B/Yamagata lineage strain was confirmed by VN assay, and for both A strains and B strains by NI assay (**Supplementary** Fig. 4**A and 5A**).

The magnitudes of the antibody responses varied depending on the serological assay performed. The HI, VN, and NI titer results for all strains were generally consistent among the age groups evaluated.

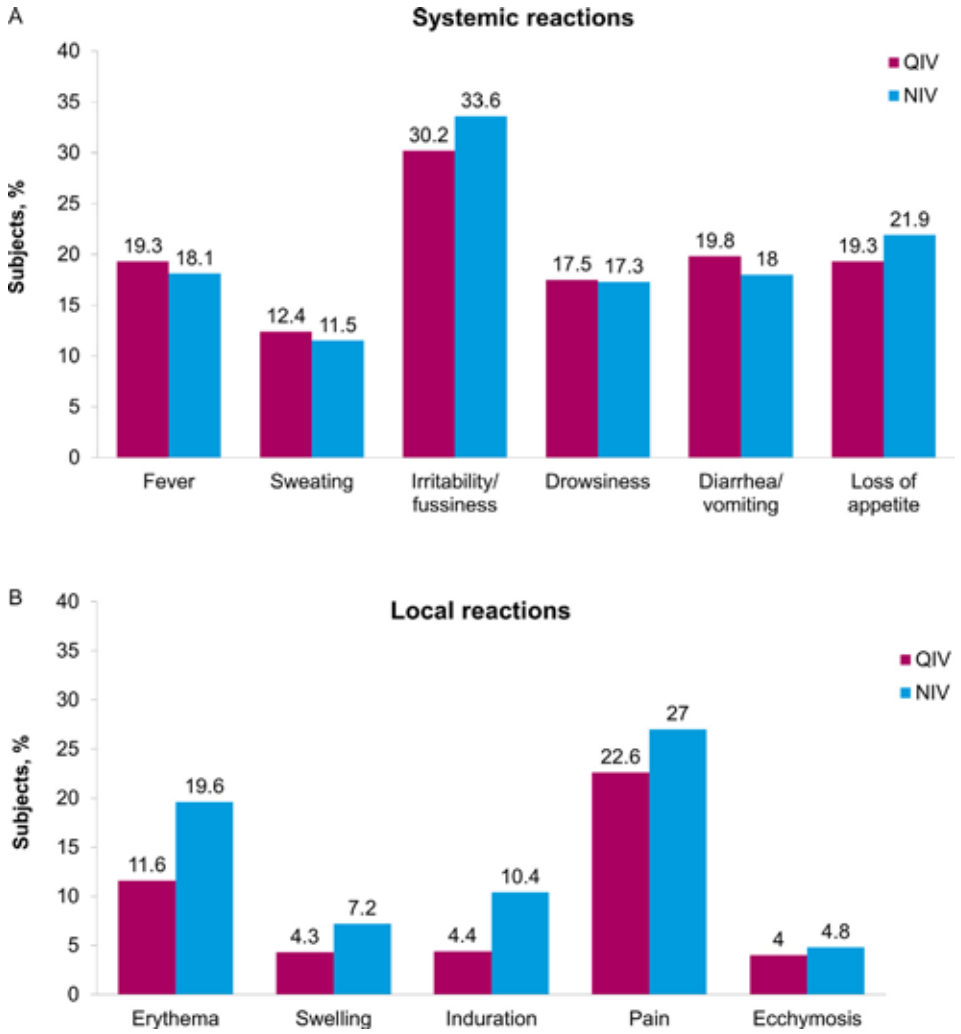

Fig. 4. Summary of reactogenicity after vaccination showing A) systemic reactions and B) local reactions. CV, control vaccine; QIV, quadrivalent influenza vaccine.

Table 2
Summary of AEs reported during the primary immunization and revaccination periods. AE, adverse event; E, number of events; QIV, quadrivalent influenza vaccine; SAE, serious adverse event; TEAE, treatment-emergent adverse event.

	Primary immunization period		Revaccination period
	QIV (n = 1005)	Control vaccine (n = 995)	QIV (n = 334)
Subjects with ≥1 TEAE, n (%) E	631 (62.8) 1815	655 (65.8) 2026	157 (47.0) 357
Subjects with ≥1 severe TEAE, n (%) E	17 (1.7) 28	14 (1.4) 23	2 (0.6) 2
Subjects with ≥1 TEAE with a reasonable possibility for causal relationship, n (%) E	21 (2.1) 29	24 (2.4) 32	1 (0.3) 1
Subjects with ≥1 SAE, n (%) E	37 (3.7) 55	54 (5.4) 79	1 (0.3) 1
Subjects with ≥1 TEAE leading to study termination, n (%) E	1 (0.1) 1	1 (0.1) 1	0
Number of deaths, n (%) E	0	0	0

3.4.3. Revaccination immune response

In participants who were revaccinated with QIV in Year 2, pre-revaccination (Visit 4) antibody titers were slightly higher for the A strains than the B strains but were generally low. Revaccination with QIV elicited strong antibody responses; HI assay demonstrated that post-revaccination (Visit 5) antibody titers were markedly higher than pre-revaccination antibody titers for all four strains (Fig. 3**B**). This response was also confirmed for all strains using VN and NI serological assays (**Supplementary** Fig. 4**B and 5B**). The HI, VN, and NI titer results for all strains were generally consistent among the age groups evaluated.

3.5. Safety

During the primary immunization period, AEs were comparable between the vaccination groups. The majority of AEs were mild to moderate in severity and no deaths were reported (Table 2). The proportion of participants with at least one treatment-emergent AE (TEAE) was similar between the QIV group (62.8%) and the control group (65.8%). The most commonly reported TEAEs overall (>5% in any vaccination group) were influenza-like illness (n = 862, 43.1%), upper respiratory tract infection (n = 139, 7.0%), rhinitis (n = 131, 6.6%), bronchitis (n = 104, 5.2%), gastroenteritis

(n = 104, 5.2%), and nasopharyngitis (n = 101, 5.1%). Two participants experienced TEAEs that led to study termination, which were one case of food allergy in the QIV group and one case of pneumonia in the control group; both were mild and were not considered related to the study vaccine. The most common TEAEs, SAEs, MAEs, AESIs, and NCIs that were reported during the primary immunization are summarized by treatment group in **Supplementary Table S4**.

Overall, 91 participants (4.6%) had at least one SAE during the primary immunization period and reporting rates were comparable between groups; 37 children (3.7%) and 54 children (5.4%) in the QIV and control groups, respectively (Table 2). The most frequent SAEs across both groups (>0.5%) were influenza-like illness (n = 17, 0.9%), pneumonia (n = 12, 0.6%), and gastroenteritis (n = 11, 0.6%). A similar number of subjects in each group had at least one MAE (QIV n = 462, 46.0%; control vaccine n = 501, 50.4%). AESIs were reported in approximately 10% of subjects in both groups (QIV n = 102, 10.1%; control vaccine n = 103, 10.4%). The most frequent AESIs (>1%) were (acute) otitis media and pneumonia. Less than 1% of subjects in both groups developed NCIs (QIV n = 8, 0.8%; control vaccine n = 7, 0.7%). The most common NCIs (>1 subject) were asthma, allergic rhinitis, and adenoidal hypertrophy.

Systemic reactions within 7 days of vaccination were comparable between groups (Fig. 4**A**) and were less common after the second dose of vaccine than after the first dose in both the QIV and control groups. Children receiving QIV had fewer local reactions than those receiving control vaccines (Fig. 4**B**) and there was no notable difference in local reactogenicity between the first and second doses of vaccine in both groups with the exception of injection-site pain, which was more severe and lasted longer for a higher proportion of subjects after the first vaccine dose *versus* the second.

The incidence of AEs was lower after revaccination with QIV compared with that during the initial immunization period (Table 2).

4. Discussion

This Phase III study evaluated the efficacy and safety of an inactivated (surface antigen) QIV in influenza-naïve children aged 6–35 months. It was conducted across three influenza seasons, during which both the circulating influenza strains and the vaccine strains varied. The absolute VE of QIV in the prevention of symptomatic influenza infection due to any circulating seasonal influenza strain, as well as antigenically matching strains, was clearly demonstrated and was generally consistent among the age groups evaluated and persistent over the surveillance period. The exception was the 6–11 months age group, which did not demonstrate VE for the prevention of influenza due to any circulating seasonal influenza strain; however, it should be noted that the study was not powered to draw firm conclusions on subgroup analyses, and there was a relatively high degree of mismatch with the influenza strains in the vaccine in this age group. The efficacy of QIV against antigenically matching influenza strains was also demonstrated consistently across the three influenza seasons evaluated. Efficacy was slightly higher among subjects in the NH compared with the SH, which may have been due in part to a lower number of antigenically matching influenza cases in the SH.

VEs similar to those demonstrated in this study have been reported for QIVs in other studies. A recent trial conducted in >12 000 children aged 6–35 months compared the efficacy of an inactivated QIV with a control vaccine over five influenza seasons and reported a VE for QIV of 64% (97.5% CI: 53, 73) for laboratory-confirmed influenza of moderate-to-severe intensity and 50% (97.5% CI: 42, 57) for laboratory-confirmed influenza of any severity in the total vaccinated cohort [28]. Another study conducted in children aged 6–35 months during the NH 2014/2015 and 2015/2016 and SH 2014 and 2015 influenza seasons reported a VE of QIV of 50.98% (97% CI: 37.36, 61.86) against influenza caused by any A or B strain and 68.40% (97% CI: 47.07, 81.92) against influenza caused by vaccine-like strains [29]. The VE demonstrated in this study was also consistent with that reported for older children: in a study assessing VE for a QIV in participants aged 3 to 8 years of age, VE was reported to be 59.3% (95% CI: 45.2, 69.7) [26], while another investigation in children aged 2 to less than 18 years of age reported a VE of 54.6% (95% CI: 45.7, 62.1) [27].

These results demonstrate that the VE of QIV in the prevention of influenza infection was high for any circulating seasonal influenza strain as well as antigenically matching influenza strains. In the current study, antigenic typing confirmed a near complete mismatch for the A(H3N2) strains. Nonetheless, the overall VE estimate was still high and comparable with the VEs found in other studies, potentially due to a degree of cross-protection against mismatched A(H3N2) strains [26–29].

An important objective of the present study was to characterize the immunogenicity of QIV in terms of initial and long-term (approximately 12 months after the first vaccination) serological response to the primary immunization, as well as in response to revaccination. Primary vaccination with QIV induced a broad serological response, characterized by elevated antibody titers. The HI data demonstrated that vaccination with QIV, but not control vaccine, induced serological responses against each of the influenza strains with results for the VN and NI assays showing a similar trend. This was accompanied by relatively high seroconversion rates (>65%). Together, these data from the NH 2017/2018 season show that vaccination with QIV induces biologically functional antibodies able to neutralize the virus as well as the production of antibodies against neuraminidase.

Approximately 12 months after primary vaccination with QIV, mean post-vaccination antibody titers remained moderately higher than pre-vaccination titers for some strains in all serological assays performed, indicating long-term persistence of immunogenicity, although the magnitude of the specific persistence varied depending on the serological assay performed. The observation that antibody responses diminish over time supports the rationale for revaccination of subjects after 12 months as recommended by the WHO [1]. Indeed, revaccination of a group of participants with QIV approximately 12 months after the primary immunization elicited a strong antibody response for all strains and in all serological assays performed. These responses were generally stronger and more pronounced than antibody responses elicited by primary vaccination for all strains across all serological assays and are indicative of the boosting effect of revaccination.

The overall safety profile and reactogenicity of QIV was comparable with those of the control vaccines. The majority of AEs were mild to moderate in severity and no deaths were reported. Systemic reactions were comparable between groups and children receiving QIV had fewer local reactions than those receiving a control vaccine. The incidence of AEs was lower after revaccination with QIV compared with the primary immunization period and, apart from injection-site pain, there was no notable difference in local reactogenicity between the first and second vaccine doses.

A major strength of the study was the ability of the results to represent three influenza seasons in Europe and Asia thereby minimizing the influence of differences in seasonal circulation of influenza strains and potential antigenic mismatches with strains included in the vaccine. Limitations were that the study was not powered to draw firm conclusions on subgroup analyses (e.g. efficacy by season, age, and strain) and, therefore, those results should be interpreted with caution. A further limitation was that the find-

ings regarding safety and reactogenicity after revaccination may be biased since not all subjects who participated in the first year continued in the study to receive revaccination in Year 2. In addition, some control vaccines are more reactogenic than flu vaccine, there were slight differences in the volume and mode of administration of some of the control vaccines, and the revaccination period was open label.

In conclusion, this study shows that primary vaccination with QIV is well tolerated and effective in protecting children aged 6–35 months against any circulating seasonal influenza strain and antigenically matching strains. The broad and persistent serological responses demonstrated in this study, including antibodies against hemagglutinin, neuraminidase, and neutralizing antibodies, support the existing evidence which shows that QIV can provide a robust immune response against key influenza strains that cause significant morbidity in young children. QIV may represent a valuable additional option for healthcare professionals when considering influenza vaccination in infants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Serge van de Witte and Jos Nauta declared that they are employees of Abbott Healthcare Products B.V.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.02.088.

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SHORT COMMUNICATION

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Detection of antibodies against influenza D virus in swine veterinarians in Italy in 2004

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Abstract

Influenza D virus (IDV) was first isolated from a swine with respiratory disease symptoms in 2011 in the United States. Epidemiological and serological studies support the hypothesis that cattle represent the natural reservoir of IDV with periodical spillover events to other animal hosts. Little is known about the seroprevalence in humans and in specific target groups such as veterinarians in Italy. This study was designed to assess the prevalence of antibodies against two influenza D lineages (D/660 and D/OK) in Italy in archived serum samples from veterinarians working with swine collected in 2004. Serum samples were tested by haemagglutination inhibition (HI) and virus neutralization (VN) assays. Results showed that 4.88% (4/82) of tested samples were positive for D/660 and 2.44% (2/82) for D/OK by HI assay. Three out of four samples showed positivity when tested by VN assay. Our data suggest undetected IDVs might have circulated and/or been introduced in Italy as early as 2004 at least in some animal species such as swine. In addition, it seems that the virus was circulating among veterinarians before the first isolation in 2011. This finding highlights the importance to continue monitoring the IDV spread in animals and humans for more detailed surveillance.

KEYWORDS
influenza D viruses, Italy, veterinarians

1 | INTRODUCTION

Influenza D virus (IDV) is a novel influenza virus isolated from a swine with respiratory disease symptoms in 2011 in the United States.[1] IDV, as influenza C virus (ICV), has seven RNA segments and only one major surface glycoprotein, the hemagglutinin-esterase-fusion, which is responsible for binding, receptor destroying, and fusion. The homology of amino acid sequences between IDV and ICV is roughly 50%, however, the distance between the two influenza viruses is similar to the one found between influenza A and B viruses (IAVs, IBVs).[2,3] No cross-reactivity has been detected between IDV and ICV.[4]

Although the virus has been first isolated in swine, several epidemiological and serological studies support the hypothesis that cattle represent the natural reservoir of IDV with periodical spillover events to other animal hosts (i.e., camel, sheep, swine, horse, goat).[4–6] The viral genome has been detected in some animal species while only specific antibodies have been detected

in horses without evidence of viral genome or virus isolation.[7] So far, there is no evidence of IDV infection in chickens and turkeys.[8] The virus has been identified in different countries across the world (i.e., France, Italy, Luxemburg, Canada, Mexico, China, Mississippi, Japan, Nebraska).[5,6,9–12]

To date, different lineages have been identified, D/OK-, D/660-, D/Japan-lineages (D/Yama2016 and D/Yama2019) and the recently identified D/CA2019.[13] D/OK and D/660 are the two major circulating lineages in North America and Europe. Until 2017, the only circulating lineage in Italy was D/OK, after phylogenetic analysis, the D/660 has been detected showing co-circulation of both lineages in the Italian cattle population.[14]

IDV seroprevalence in different animal species has been assessed in Italy. Cattle show to have a high prevalence ranging from 92.4% to 74% (active and active/passive surveillance, respectively). Regarding swine from Northern Italy, the seroprevalence value was from 0.6% to 11.7% depending on the year of sampling (2009–2018). Low prevalence has been detected in wild boars from the Alpine and Northern Apennine areas (1.92%, 2018–2019) and in wild ungulates (0.98%). Sheep and goats show a prevalence of 6.3% and 3.1%, respectively, in 2016–2017.[15,16]

The live trade seems to play a key role in viral spread considering that Italy, together with Spain, is one of the most important cattle importers in Europe from France. Data report higher IDV seroprevalence in importing countries (i.e. Italy) than in exporting countries suggesting that cattle may come in contact with the virus during transportation or just after.[15]

IDV seroprevalence in Italy has been studied on general population only, ranging from 5.1% in 2005 to 46.0% in 2014.[17] International studies on cattle and farming workers performed in Florida and Malaysia showed a seroprevalence of 94% and 1.3%, respectively, suggesting that cattle-exposed people could be infected with IDV through occupational zoonotic transmission.[18,19]

The aim of this study was to assess the prevalence of antibodies against two IDV lineages (D/660 and D/OK) in Italy in archived serum samples from veterinarians working with swine collected in 2004.

2 | MATERIALS AND METHODS

2.1 | Influenza viruses

Influenza D/bovine/Oklahoma/660/2013 (D/660) and influenza D/OK -D/swine/Italy/199724/2015 (D/OK) viruses were propagated in Madin-Darby Canine Kidney (MDCK) cells as previously described.[17]

2.2 | Serum samples

A total of 82 serum samples were collected from a group of Italian veterinarians working with swine and attending the 30th meeting of the Italian Society of Pathology and Breeding of Pigs (SIPAS) in 2004. The enrolled veterinarians worked in Northern and Central Italy, an area with the highest density of pigs and swine farms.

The median age of the study population was 41 years, with a range of 24–76 years; 76.8% of samples were from male subjects.

Influenza D (swine) hyperimmune serum against D/swine/Italy/199724/2015 was used as the positive control.

Influenza C, influenza A (H1N1 and H3N2), influenza B (Victoria and Yamagata lineages) hyperimmune serum samples were included as controls in the assay.

Human serum without immunoglobulin A, immunoglobulin M, and immunoglobulin G was used as a negative control (Sigma-Aldrich).

All serum samples were tested by the haemagglutination inhibition (HI) assay.

Positive samples with a sub-set of negative samples were tested by the virus neutralization (VN) assay.

2.3 | HI assay

The HI assay was performed as previously described.[17] All serum samples, including positive and negative controls, were pretreated with receptor-destroying enzyme (ratio 1:5) from Vibrio cholerae (Sigma-Aldrich) for 18 h at 37°C in a water bath followed by heat inactivation for 1 h at 56°C in a water bath with 8% sodium citrate (ratio 1:4). All serum samples were tested in duplicate using turkey red blood cells (0.35%). The antibody titer was expressed as the reciprocal of the highest serum dilution showing complete inhibition of agglutination. Since the starting dilution was 1:10, a titer below the detectable threshold was conventionally expressed as 5 for calculation purposes.

2.4 | Virus neutralization

The MDCK cell cultures were grown at 37°C in 5% CO_2 and preincubated for 4 h.

Serum samples, including positive and negative controls, were previously heat-inactivated at 56°C for 30 min. Samples twofold diluted with EMEM culture medium supplemented with 0.5% fetal bovine serum were mixed with an equal volume of virus (100 TCID50/well). After 1 h of incubation at 37°C in 5% CO_2, 100 µl of the mixture was transferred to a plate containing 1.5×10^4 MDCK cells/well. Plates were read for haemagglutination activity in the supernatant after 5 days of incubation at 37°C in 5% CO_2. The VN titer was expressed as the reciprocal of the highest serum dilution showing the absence of haemagglutination.

2.5 | Statistical analysis

Seroprevalence rates were calculated along with the corresponding 95% confidence interval (CI) using the adjusted Wald method (GraphPad QuickCalc, https://www.graphpad.com/quickcalcs/).

TABLE 1 HI and VN titers of veterinarians samples collected in 2004 by tested lineage

Sample	Age (years)	HI assay		VN assay	
		D/660	D/OK	D/660	D/OK
1	42	10–10	5	40	20
2	28	80–40	5	40	10
3	39	80–80	80–80	ND	ND
4	50	40–80	20–20	40	20

Abbreviations: HI, haemagglutination inhibition; ND, not determined, VN, virus neutralization.

TABLE 2 HI titers of hyperimmune serum samples included as controls in the assay

Assay control	IDV lineages	
	D/660	D/OK
IDV (D/OK)	1280	10240
ICV	5	5
H1N1	5	5
H3N2	5	5
B Victoria	5	5
B Yamagata	5	5

Note: IDV: hyperimmune serum against D/swine/Italy/199724/2015 virus; ICV: hyperimmune serum against C/Victoria/2/2012 virus; H1N1: hyperimmune serum against A/Michigan/45/2015 (H1N1) virus; H3N2: hyperimmune serum against A/Hong Kong/45/2015 (H3N2) virus; B Victoria: hyperimmune serum against B/Brisbane/60/2008 virus; B Yamagata: hyperimmune serum against B/Phuket/3073/2013 virus.

3 | RESULTS

Out of 82 samples tested, 4 samples (4.88%, 95% CI: 1.54–12.26) showed HI positivity for D/bovine/Oklahoma/660/2013 strain (D/660-lineage), two of them (2.44%, 94% CI: 0.15–8.98) were positive for D/swine/Italy/199724/2015 strain (D/OK-lineage) as well. The HI levels of positivity range from 10 to 80 for D/660 and from 20 to 80 for D/OK (Table 1).

Three out of 4 samples were further tested by the VN assay and showed positivity for both lineages with titers ranging from 10 to 40 (Table 1). Unfortunately, we did not have enough serum for one sample to be tested by the VN assay (Table 1).

Table 2 shows the HI titers of all controls against D/bovine/Oklahoma/660/2013 strain (D/660-lineage) and D/swine/Italy/199724/2015 strain (D/OK-lineage).

4 | DISCUSSION

In this study, we tested 82 archived serum samples from swine veterinarians, working in Northern and Central Italy, collected in 2004 and 4.8% had HI titers ≥10 for D/660. This positive result suggested that there might have been undetected IDV circulation or introduction in Italy as early as 2004. Our previous study conducted in Italy from 2005 to 2017 has shown that 5.1% of the general population had antibodies against D/660 in 2005. International studies have detected IDV or antibodies against IDV in cattle workers in Florida in 2011–2012,[18] in humans recruited in Canada and Connecticut during 2007–2008 and 2008–2009 influenza seasons,[1] in animal workers in Malaysia in 2017,[19] in bioaerosol sampling in North Carolina in 2017–2018 and in 2018.[20,21] Notably, two studies performed in animals revealed antibodies specific for IDV in one goat sample collected in April 2002 in Massachusetts and in the Mississippi cattle population since at least 2004,[8,9] highlighting the possibility that the virus was circulating among some animal species before the first isolation in 2011.

The HI-positive samples were further tested by the VN assay. All samples positive for D/660 were confirmed by the VN assay. Surprisingly, two samples tested negative by HI for D/OK, showed measurable antibody titers, though low, by the VN assay. The same inconsistency has been detected in another study[8] conducted in animal serum samples providing two possible explanations. The first one might be the more sensitive nature of the VN assay for detecting antibodies. The other one could be the ability of the VN assay to detect functional antibodies different from the ones detected by the HI assay. Overall, based on the VN results, the seroprevalence provided by the HI assay may be slightly underestimated.

Based on the HI and VN data, we found human samples positive for both lineages, D/660 and D/OK. Serological data on animal samples found low positivity for D/OK in 2009 and a steady increase from roughly 2015 in Italy.[22] In addition, it seems that up to 2017, all the Italian IDVs isolated belonged to the D/OK genetic cluster and the earliest D/660 strains were detected in 2018 from cattle imported from France.[14] These findings might appear to be in contrast with our results. However, it should be pointed out that further investigations on animal samples, sera, and swabs, coming from different animal species and geographic areas, are needed to better understand and explore IDVs circulation and/or introduction in Italy. We can hypothesize undetected introduction of D/660 in animals, particularly in swine based on our data, with an undetected animal outbreak and that maybe the virus has started to steadily circulate in recent years only. On the other hand, as the VN titers for D/OK are slightly lower than those for D/660, it is possible that the exposition to one IDV can induce cross-reactive antibodies to the other lineages. Basically, D/660 might have been circulated in Italy before its detection, however, those assumptions need to be confirmed by further studies.

This study has some limitations. First of all, the number of tested samples is small, and they belong to swine veterinarians only. In addition, there are no animal samples collected in 2004 in

the same geographic area to be tested. One HI-positive sample was not enough to be tested by the VN assay and consequently, the seroprevalence might be slightly underestimated. The samples were not tested for the presence of antibodies against ICV. So far, serological studies did not detect cross-reactivity with antibodies directed against human IAV, IBV, and ICV. However, as ICV is a ubiquitous human pathogen, further studies are needed, supported by the development of a virus-specific assay able to accurately evaluate IDV antibody prevalence in human subjects.[16,23]

Overall, our findings on human serum samples might have two implications. The first one is that undetected IDVs, D/660 and/or D/OK, might have circulated and/or been introduced in Italy as early as 2004 at least in some animal species such as swine. To support these findings and draw definitive conclusions on when IDV has been introduced in Italy and start to circulate, infect, and be transmitted among animals and potentially to humans, it would be important to analyze more archived samples. In particular, samples from animal species susceptible to IDVs infection and from humans, especially people working with animals and those exposed to cattle, covering wide Italian geographic areas. The second one is related to a public health perspective. The data in this study provide further insights on the ability of IDVs to infect and elicit an immune response in humans and should be evaluated considering several aspects. Basically, influenza viruses are characterized by an evolving nature. There is evidence of interspecies transmission and the international appearance of IDV in the animal population worldwide. In addition, IDV can infect ferrets, the gold standard for influenza studies in animals, and guinea pigs, and can replicate efficiently in a human airway epithelium model.[1,15] Considering all the above and the lesson learned from SARS-CoV-2, it would be key not to underestimate the IDV potential as a threat for humans or at least for specific target groups and to continue monitoring IDVs spread in animals and humans.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTION

Conceptualization: Claudia Maria Trombetta; *Formal analysis*: Serena Marchi, Fabio Ostanello; *Investigation*: Claudia Maria Trombetta, Serena Marchi; *Resources*: Ilaria Di Bartolo, Fabio Ostanello, Chiara Chiapponi; *Writing—original draft preparation*: Claudia Maria Trombetta; *Writing—review and editing*: Emanuele Montomoli, Ilaria Di Bartolo, Fabio Ostanello, Chiara Chiapponi, Serena Marchi; *Visualization*: Serena Marchi; Project administration: Claudia Maria Trombetta.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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vaccines

MDPI

Article

Correlation of Influenza B Haemagglutination Inhibiton, Single-Radial Haemolysis and Pseudotype-Based Microneutralisation Assays for Immunogenicity Testing of Seasonal Vaccines

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Abstract: Influenza B is responsible for a significant proportion of the global morbidity, mortality and economic loss caused by influenza-related disease. Two antigenically distinct lineages co-circulate worldwide, often resulting in mismatches in vaccine coverage when vaccine predictions fail. There are currently operational issues with gold standard serological assays for influenza B, such as lack of sensitivity and requirement for specific antigen treatment. This study encompasses the gold standard assays with the more recent Pseudotype-based Microneutralisation assay in order to study comparative serological outcomes. Haemagglutination Inhibition, Single Radial Haemolysis and Pseudotype-based Microneutralisation correlated strongly for strains in the Yamagata lineage; however, it correlated with neither gold standard assays for the Victoria lineage.

Keywords: influenza B viruses; serology

1. Introduction

Influenza is a respiratory syndrome caused by three of six genera in the Orthomyxoviridae family, influenza A, B and C. A fourth genus (Influenza D) has also been characterised [1]. Influenza A virus is the most widespread; its various subtypes are classified according to their antigenically variable surface glycoproteins: haemagglutinin (HA, H1-H18) and neuraminidase (NA, N1-N11).

Influenza B viruses comprise two co-circulating, antigenically distinct lineages that diverged from their progenitor, strain B/Hong Kong/8/1973, into the "Yamagata-like" (B/Yamagata/16/1988 type) and "Victoria-like" (B/Victoria/2/1987 type) lineages [2]. This human virus causes a significant proportion (20–30%) of global morbidity associated with influenza virus disease due to its global distribution and unpredictable switches in the predominating lineage circulating [3–5]. The WHO vaccine recommendations include an up-to-date strain from both lineages for quadrivalent vaccines, but only one for trivalent vaccines. Should the circulating lineage not match the predicted lineage, there is an inevitable drop in coverage against influenza B-linked disease [3,6–8]. In the United States, multiple quadrivalent vaccines have been approved and are in use [9].

1.1. Serological Assays for Influenza B

Single Radial Immuno-Diffusion (SRID) has been one of the mainstays for the identification and characterisation of inactivated influenza vaccines, correlating with immunogenicity and clinical benefit/protection [10–17]. Haemagglutination Inhibition (HI) has been used for many decades as the tool used for the detection of influenza antibodies [18]. These assays, in combination with ELISA [19] and Single Radial Haemolysis (SRH) [20] comprise the gold standard assays for detection of influenza virus targeting antibodies and are generally applicable to the B type. However, more recent work has highlighted distinct shortcomings of the traditional assays, focusing research on the development of novel assays utilising various different technologies [21–30]. Ether treatment has been employed for influenza B viruses, prior to HI experiments, raising the efficacy of the HI test to that of the Complement Fixation (CF) test [31–33]. This technique was developed due to the lack of reactivity of certain strains of influenza, as well as the B type. Alongside the adaptation of SRID to influenza B resulting in SRH, historical use of ether treatment was found to increase sensitivity but reduces the specificity of HI during assay of serum samples against influenza B [20,31,32,34–37]

1.2. Monoclonal Antibodies

Monoclonal antibodies (mAbs) are increasingly being used in influenza research, whether for development of standards to complement or validate assays, or more directly to evaluate Haemagglutinin (HA) epitopes targeted through vaccination, or as potential therapeutics, as seen in the recent Ebola outbreak [38,39]. The mAbs employed in this study were developed as an antibody-based alternative for influenza B identity (Yamagata or Victoria lineages) and potency assays [40].

1.3. Study Aims

This study's goal is determining the correlation of the gold standard assays HI and SRH with the recently adapted influenza B Pseudotype-based Microneutralisation assay (pMN), as HI detects primarily receptor binding site (RBS) proximal antibodies while SRH detects IgG1, IgG3 and IgM class antibodies that are compatible with the complement cascade. pMN detects HA-neutralising antibodies directed against both the globular head and the stalk [41]. As with SRH, HI has been correlated with protection against influenza, with titres at or above 40 linked to 50% or greater protection from infection in adults [12,42–44]. Linking either of these assays with pMN, despite the detection of different types of antibody, would allow more confidence to be attributed to the results generated using this assay, despite it actually detecting neutralisation of the function of the influenza HA glycoprotein. Correlation data between assays is important for in-depth interpretation of immunogenicity data, especially when correlation is determined with an assay that has been used for in vivo or challenge studies, establishing a correlate of protection. By inter-comparison of assays utilising different approaches, the scientific community can make more informed decisions on the future direction of vaccine design and testing. To date, no study of this type has been performed using influenza B, with efforts focusing more on the predominant A type [45,46]. As a major contributor to morbidity and mortality, it is essential to interrogate the correlations and relationships between data produced for influenza B using a range of assays. This is especially important as this type is lacking a definitive reservoir and circulates yearly as part of two lineages. In this study, influenza B lentiviral pseudotypes (PV) will be interrogated using a defined set of mAbs and a panel of sera, to put this resource to use in the correlation of pMN, SRH and HI.

2. Materials and Methods

2.1. Plasmids

Expression plasmid phCMV1 bearing the HA gene for B/Hong Kong/8/1973, B/Yamagata/16/1988 and B/Florida/4/2006 were obtained from Dr Davide Corti, Institute for Research in Biomedicine, Bellinzona, Switzerland. B/Victoria/60/2008,

B/Brisbane/60/2008 and B/Bangladesh/3333/2007 HA genes were subcloned into plasmid pI.18. Plasmids p8.91 and pCSFLW were obtained from Prof. Greg Towers, University College, London, and originate from gene therapy applications [47,48]. To achieve maturation of HA gene products, protease-encoding plasmids were used [49–52], as detailed in Table 1 alongside the quantity transfected per well of a 6-well plate. Information regarding the strains of influenza B used can also be found in Table 1.

Table 1. Influenza B strains, accession numbers, lineage, protease type and quantity required for production in 6-well format are displayed. Human Airway Trypsin (HAT) and Transmembrane protease, serine 4 (TMPRSS4) expression plasmids were co-transfected with other plasmids to allow maturation of HA0 to HA1/2 inside producer cells.

Strain	Accession	Lineage	Protease	ng per 6-Well
B/Hong Kong/8/1973	K00425	Pre-split	HAT	125
B/Victoria/2/1987	FJ766840	Victoria	HAT	125
B/Brisbane/60/2008	KX058884	Victoria	TMPRSS4	125
B/Yamagata/16/1988	CY018765	Yamagata	HAT	125
B/Florida/4/2006	EU515992	Yamagata	HAT	125
B/Bangladesh/3333/2007	CY115255	Yamagata	HAT	250

2.2. Serum Samples

One serum set was used per lineage of influenza B, consisting of samples taken pre and post seasonal vaccination. Paired low and high responders (based on HI results) were chosen. In total, 41 pairs of sera were assayed against B/Brisbane/60/2008 and 43 pairs against B/Florida/04/2006. Anonymised serum samples were obtained from Italian subjects and in compliance with Italian ethics law.

2.3. Pseudotype Production

Lentiviral pseudotypes were produced by transient transfection of HEK293T/17 cells with lentiviral packaging construct plasmids p8.91 [48] and pCSFLW [47] alongside an influenza B glycoprotein expression plasmid and corresponding protease expression plasmid as described in Section 2.1. Cells were transfected with 500 ng HA expression plasmid and corresponding protease listed in Table 1, alongside 500 ng p8.91 and 750 ng pCSFLW per well in a 6-well format. After 8 h, media was replaced and 1 unit of exogenous neuraminidase (*Clostridium perfringens*, Sigma, St. Louis, MO, USA) added per well to enable HA-pseudotype release. Cell culture supernatants were harvested 48 h post-transfection, filtered at 0.45 µm and titrated for transduction-based (firefly) luciferase activity on HEK293T/17 cells in 96-well format (Supplementary Figure S1).

2.4. Pseudotype-Based Microneutralisation Assay

Serial (1:2) dilutions of serum were performed in 96-well format in a total volume of 50 µL, and 50 µL of lentiviral pseudotype added to give a total relative luminescence (RLU) output of 1×10^6 RLU per well. Serum and virus were incubated for 1 h at 37 °C, 5% CO_2 in a humidified incubator, then 1.5×10^4 HEK293T/17 cells were added per well. Plates were then incubated at 37 °C, 5% CO_2 in a humidified incubator for 48 h, whereupon 50 µL of Bright-Glo (Promega) reagent was added per well and luminescence read after a 5 min incubation period. Data points were normalised to 100% and 0% neutralisation plate controls, and non-linear regressional analysis was performed to obtain neutralisation curves and IC_{50} and IC_{90} values. R^2 values of 0.8 or more were used as a cut-off for confidence in the titration of antibody response, and any samples below this were discarded.

2.5. Haemagglutination Inhibition Assay

The influenza viruses used in the HI were B/Brisbane/06/2008 (15/146) and B/Florida/04/2006 (08/138), obtained from the National Institute for Biological Standards and Control (NIBSC), United Kingdom. Serum samples were pre-treated with receptor destroying enzyme (RDE) from *Vibrio cholerae* (Sigma Aldrich) at 1:5 ratio for 18 h at 37 °C in a water bath and then heat-inactivated for 1 h at 56 °C in a water bath with 8% sodium citrate at a 1:4 ratio. Turkey erythrocytes (Emozoo S.N.C, Casole d'Elsa, Italy) were centrifuged twice, washed with a saline solution (0.9%) and adjusted to a final dilution of 0.35%. RDE-treated serum samples were diluted two-fold with saline solution (0.9%) in a 96-well plate, starting from an initial dilution of 1:10. 25 µL of influenza virus was added to each well, and the mixture was incubated at room temperature for 1 h. At the end of the incubation, erythrocytes were added and the plates were evaluated for the inhibition of agglutination after 1 h at room temperature.

The antibody titre was expressed as the reciprocal of the highest serum dilution showing complete inhibition of agglutination. Since the starting dilution was 1:10, the lower limit of detectable antibody titre was 10. When the titre was below the detectable threshold, the results were conventionally expressed as 5 for calculation purposes, half the lowest detection threshold. Geometric mean titers were calculated from experimental repeats.

2.6. Single Radial Haemolysis Assay

The influenza viruses B/Brisbane/06/2008 (15/146) and B/Florida/04/2006 (08/138) were obtained from the NIBSC. Serum samples were heat-inactivated at 56 °C for 30 min in a water bath. Turkey erythrocytes were centrifuged twice and washed with phosphate-buffered saline (PBS). Diluted virus was added to the erythrocyte suspension at a concentration of 2000 haemagglutination units (HAU) per mL. The erythrocyte–virus suspension was incubated at 4 °C for 20 min, and subsequently, a solution of 2.5 mM Chromium Chloride ($CrCl_3$) was added to the suspension, and it was incubated at room temperature for 10 min. The suspension was then carefully mixed once and then centrifuged. A stock solution consisting of 1.5% agarose in PBS containing 0.1% sodium azide and 0.5% low gelling agarose was prepared. This agarose stock solution was maintained at 45 °C in a water bath. The final suspension of erythrocytes, virus and guinea pig complement was vigorously shaken and evenly spread onto each plate. Plates were incubated at room temperature for 30 min and then stored at 4 °C for 30–90 min. Holes were introduced into each plate with a calibrated punch, and 6 µL of each serum sample was dispensed into each hole. The plates were stored in a humid box and incubated at 4 °C for 16–18 h in the dark. Subsequently, plates were incubated in a water bath at 37 °C for 90 min; diameters of the areas of haemolysis were then read in mm with a calibrating viewer [20,53].

2.7. mAbs and Controls

Influenza B mAbs were kindly provided by Dr Jerry P Weir, Division of Viral Products, Food and Drug Administration (FDA), USA [40]; see Table 2. Anti-B/Brisbane/60/2008 HA serum (11/136) was obtained from the NIBSC and employed against all strains of influenza B as a serum positive control. This antiserum had previously been tested in our laboratory, showing that it was capable of neutralising all available strains of influenza B to varying degrees, with the highest potency against the matched B/Brisbane/60/2008 strain [54].

Table 2. Influenza B mAbs. Five mAbs used, two specific for each lineage of influenza B and one binding to an epitope conserved between both lineages (cross).

mAb	2F11	3E8	1H4	8E12	5A1
Target lineage	Cross	Yamagata	Yamagata	Victoria	Victoria

2.8. Statistical Analysis

Mean endpoint antibody titres (or IC_{50} for pMN) for each serum sample were compared between assays. This comparison was carried out in GraphPad Prism where a two-tailed Pearson's correlation was performed on pairs of data sets that were plotted against each other. A line of best fit was used in order to show the general trend of correlation between data sets. Analysis was performed between pre- (V1) and post-vaccination (V2) sera, and fold change from V1 to V2. pMN data consisted of both IC_{50} and IC_{90} values, and further analysis was carried out on transformed (log10) V2 data.

3. Results

3.1. Lineage Specific and Cross-Reacting mAbs Neutralise Influenza B PV

Lineage-specific mAbs neutralised all influenza B PV except B/Brisbane/60/2008, which was not susceptible in our experiments. This PV was unaffected by either the Victoria specific mAbs, or the 2F11 cross-lineage mAb. Despite this, anti-B/Brisbane/60/2008 HA serum showed the highest neutralisation against the matched B/Brisbane/60/2008 pseudotypes (IC_{50} > 256,000) (Figure 1). The 2F11 mAb neutralised Yamagata lineage strains the strongest (IC_{50}: 2–4 ng/mL), followed by the pre-lineage strain B/Hong Kong/8/1973 (IC_{50}: 8 ng/mL) and finally the Victoria strain B/Victoria/2/1987 (IC_{50}: 160 ng/mL). Yamagata lineage-specific mAbs 3E8 and 1H4 were only effective on Yamagata lineage PVs, neutralising B/Bangladesh/3333/2007, the strongest (IC_{50}: 3 and 4 ng/mL, respectively), followed by B/Florida/4/2006 (IC_{50}: 21 and 3 ng/mL) and B/Yamagata (IC_{50}: 34 and 6 ng/mL). Victoria-specific mAbs 8E12 and 5A1 neutralised B/Hong Kong the strongest (IC_{50}: 2 and 4 ng/mL, respectively), followed by B/Victoria/2/1987 (IC_{50}: 47 and 22). See Table 3 for a list of IC_{50} values.

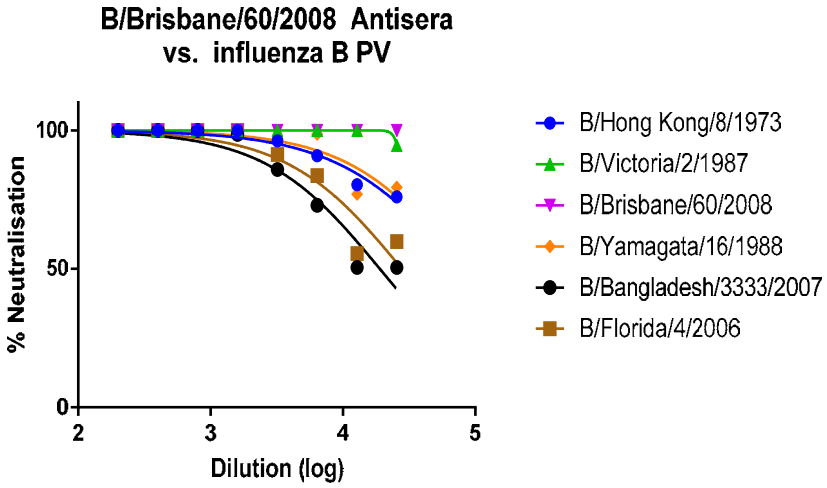


Figure 1. Anti-B/Brisbane/60/2008 HA serum (11/136, NIBSC) neutralises matched and related strains B/Brisbane/60/2008 and B/Victoria/2/1987 the strongest but also neutralises Yamagata lineage strains and the B/Hong Kong/7/1973 precursor to a lesser extent.

Table 3. IC_{50} values in ng/mL for the neutralisation of 6 strains of influenza B HA-PV by mAbs. Pre-lineage strain, Yamagata and Victoria lineages highlighted in grey, green and blue, respectively. With the exception of B/Brisbane/60/2008, the cross lineage mAb 2F11 acts as expected, neutralising all influenza B PV. Yamagata specific mAbs neutralise PV bearing HAs from Yamagata lineage strains, while Victoria-specific mAbs neutralise PV bearing HAs from Victoria lineage strains with the exception of B/Brisbane/60/2008. The pre-lineage spli t strain B/Hong Kong/8/1973 is neutralised in a Victoria-like manner by 2F11 and Victoria lineage-specific mAbs 8E12 and 5A1.

mAb	Target	B/Hong Kong/8/1973	B/Florida/4/2006	B/Bangladesh/3333/2007	B/Victoria/2/1987	B/Brisbane/60/2008
2F11	Cross	8	3	2	4	-
3E8	Yamagata	-	34	21	3	-
1H4	Yamagata	-	6	3	4	-
8E12	Victoria	2	-	-	-	-
5A1	VIctoria	4	-	-	-	-

Anti B/Brisbane/60/2008 polyclonal sheep serum was used as an anti-influenza B control in all of these experiments and for all strains of influenza B PV due to its ability to neutralise all strains tested in the pMN assay, regardless of lineage. This antiserum is most potent against the homologous strain B/Brisbane/60/2008 and the other Victoria lineage strain B/Victoria/2/1987 (Figure 4), and 100% neutralisation is seen from 1:200 up to the penultimate dilution point 1:128,000.

The data for the Victoria lineage viruses show reduced antibody potency, with IC_{50} values up to 20 times higher. Victoria epitope targeting mAbs neutralise B/Victoria as well as the Hong Kong precursor. Neutralisation curves for the above data are shown in Figures 2–4.

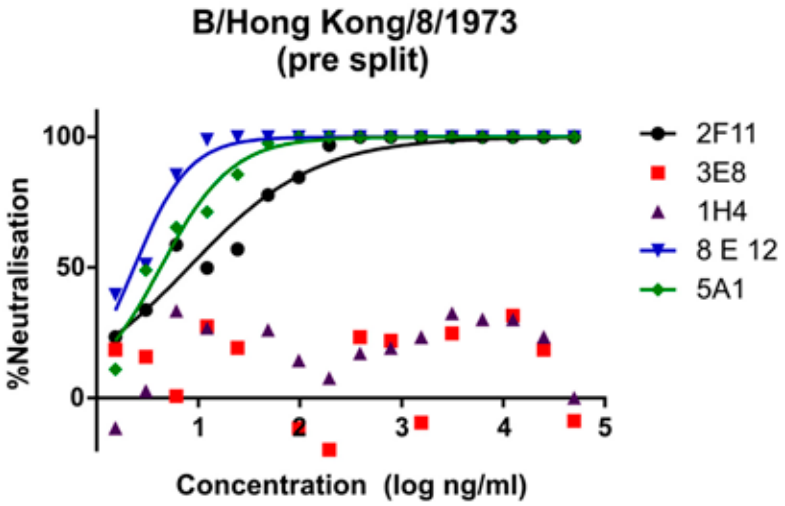


Figure 2. pMN neutralisation curves for FDA mAbs 2F11, 3E8, 1H4, 8E12 and 5A1 against PV bearing the HA glycoprotein from the influenza B pre-lineage split strain, B/Hong Kong/8/1973. This PV is neutralised strongly by Victoria-specific mAbs 5A1, 8E12 and cross-lineage mAb 2F11 but not Yamagata specific mAbs 3E8 or 1H4.

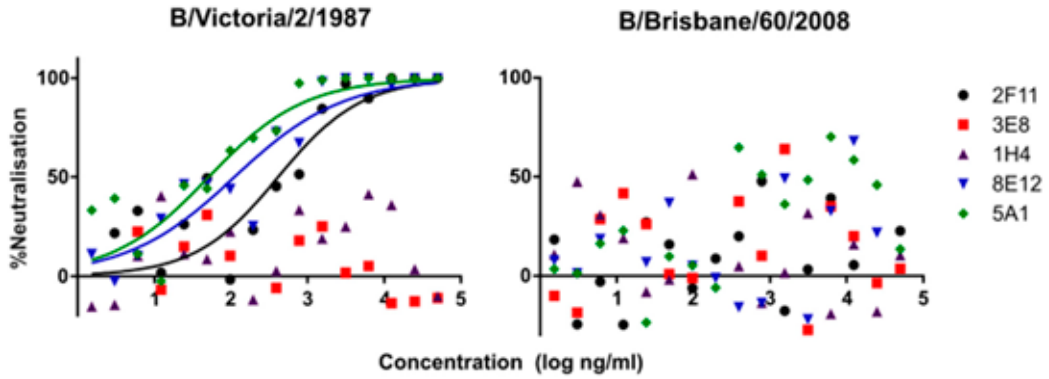


Figure 3. pMN neutralisation curves for FDA mAbs 2F11, 3E8, 1H4, 8E12 and 5A1 against PV bearing HA glycoproteins from Victoria lineage strains B/Victoria/2/1987 and B/Brisbane/60/2008. Victoria lineage PVs are not neutralised by Yamagata-specific mAbs 3E8 or 1H4. A/Victoria/2/1987 PV is neutralised by Victoria lineage-specific mAbs 5A1, 8E12 and cross-lineage mAb 2F11. B/Brisbane/60/2008 PV are not neutralised by any of the mAbs tested, including Victoria lineage and cross-lineage mAbs with a high degree of assay variability.

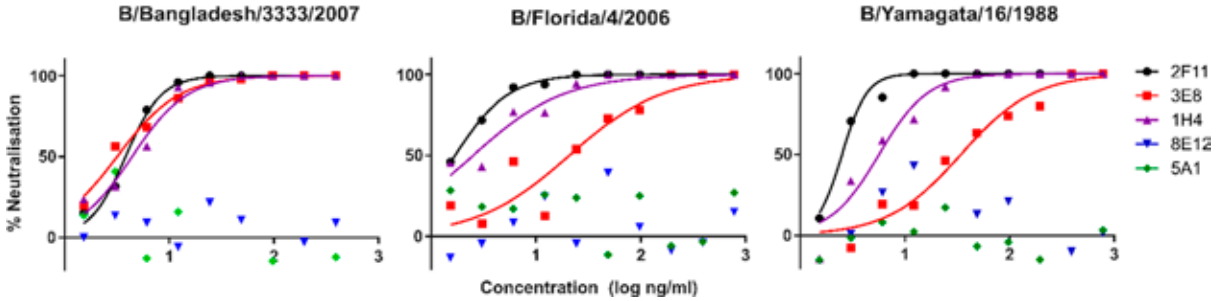


Figure 4. pMN neutralisation curves for FDA mAbs 2F11, 3E8, 1H4, 8E12 and 5A1 against PV bearing HA glycoproteins from Yamagata lineage strains B/Yamagata/16/1988, B/Florida/4/2006 and B/Bangladesh/3333/2007. Potent neutralisation by Yamagata specific mAbs 3E8 and 1H4, and by cross-lineage mAb 2F11. No neutralisation is observed for Victoria lineage-specific mAbs 8E12 and 5A1.

3.2. Correlation of SRH, HI and pMN

SRH, HI and pMN data for strains B/Florida/04/2006 (Yamagata) and B/Brisbane/60/2008 (Victoria) were analysed to assess the correlation between each assay. Endpoint titres for high responders (V2) are displayed in Figure 5. The titre profiles of each serum set are very similar between the two viruses, despite each virus having its own unique set of sera. HI values range from 5 to 1280 for both B/Brisbane/60/2008 and B/Florida/04/2006, with arithmetic means at 156 and 270, respectively. SRH values range from 37 to 106 and 38 to 99 mm^2 for B/Brisbane/60/2008 and B/Florida/04/2006, respectively. Arithmetic means were 58 and 57 mm^2, respectively. pMN (IC_{50}) values ranged from 4594 to 209,395 for B/Brisbane/60/2008 and 3349 to 31,954 for B/Florida/04/2006. Arithmetic mean pMN titre means were 41,613 and 11,740, respectively.

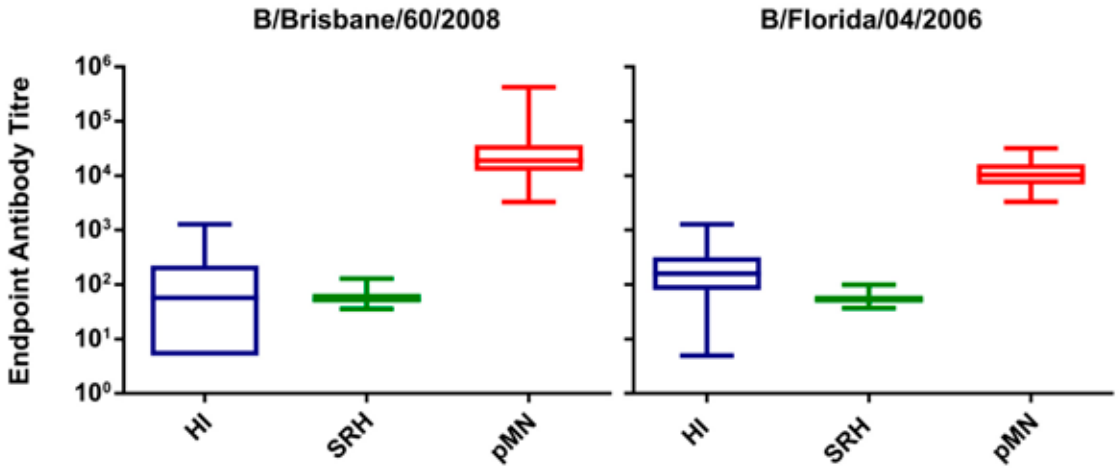
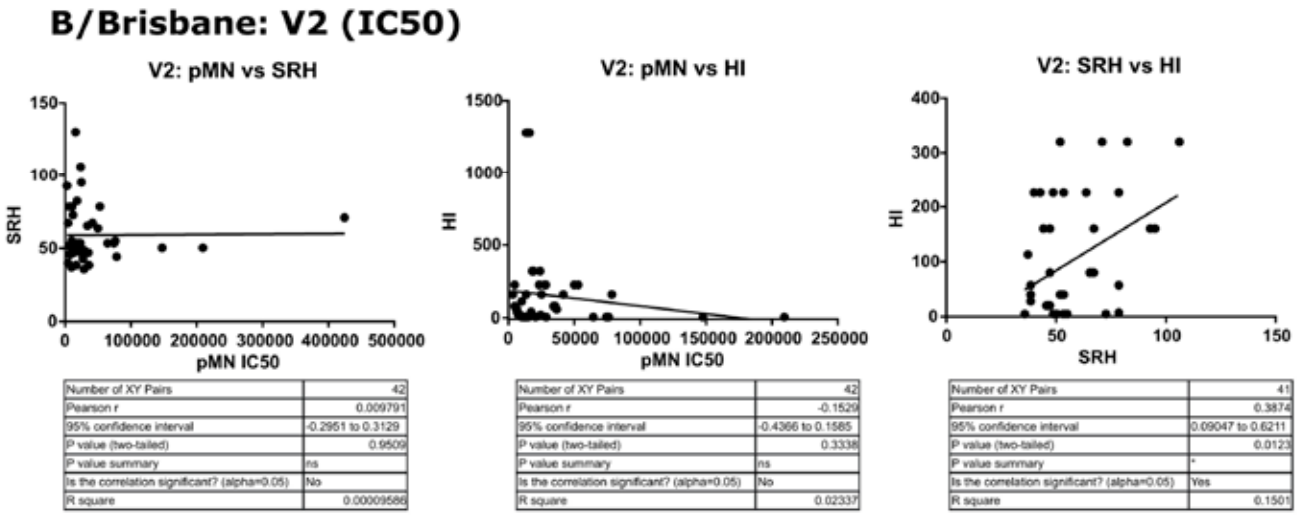


Figure 5. Haemagglutination Inhibition (HI), Single Radial Haemolysis (SRH) endpoint titres and pMN IC_{50} values for high responders (V2) against B/Brisbane/60/2008 and B/Florida/04/2006. Titres displayed show similar profiles for each strain tested, with a broader range of pMN IC_{50} values for B/Brisbane/60/2008.

3.3. Correlation of Data: B/Brisbane/60/2008 IC_{50}

Two different sets of values for each assay were correlated, V2 data (post-vaccination high responders) and fold change between V1 and V2. For B/Brisbane/60/2008, no correlation was observed between pMN and the other assays, with Pearson's r values ranging from −0.07 to −0.08 (p = 0.13 to 0.9). SRH and HI correlated when fold changes were compared (r = 0.224, p = 0.1539) and increased after the removal of two outliers (r = 0.60, $p \geq 0.0001$). V1 results weakly correlated (r = 0.32, p = 0.0375) for SRH and HI, and more so for V2 results (r = 0.39, p = 0.0123). See Figures 6 and 7 for correlation graphs and data.



Number of XY Pairs	42
Pearson r	0.009791
95% confidence interval	-0.2951 to 0.3129
P value (two-tailed)	0.9509
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R square	0.00009586

Number of XY Pairs	42
Pearson r	-0.1529
95% confidence interval	-0.4366 to 0.1585
P value (two-tailed)	0.3338
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R square	0.02337

Number of XY Pairs	41
Pearson r	0.3874
95% confidence interval	0.09047 to 0.6211
P value (two-tailed)	0.0123
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R square	0.1501

Figure 6. Correlation of SRH, HI and pMN (IC_{50}) mean V2 values assayed against B/Brisbane/60/2008 PV. Pearson's two-tailed analysis performed using GraphPad Prism. No correlation was observed between IC_{50} antibody titres and SRH or HI (Pearsons r = 0.009 and −0.152, respectively). SRH and HI values were correlated (Pearsons r = 0.38). $p > 0.05$ = ns, $p \leq 0.05$ = *.

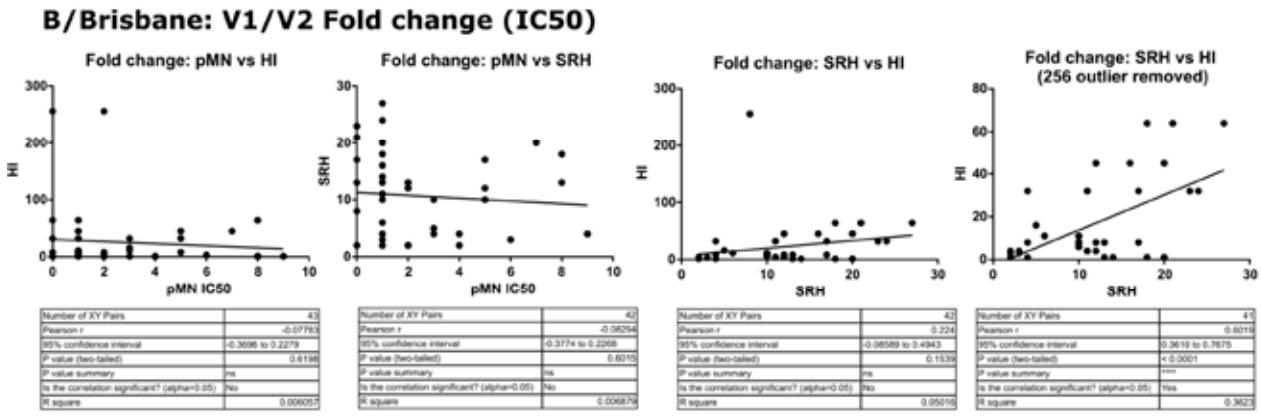
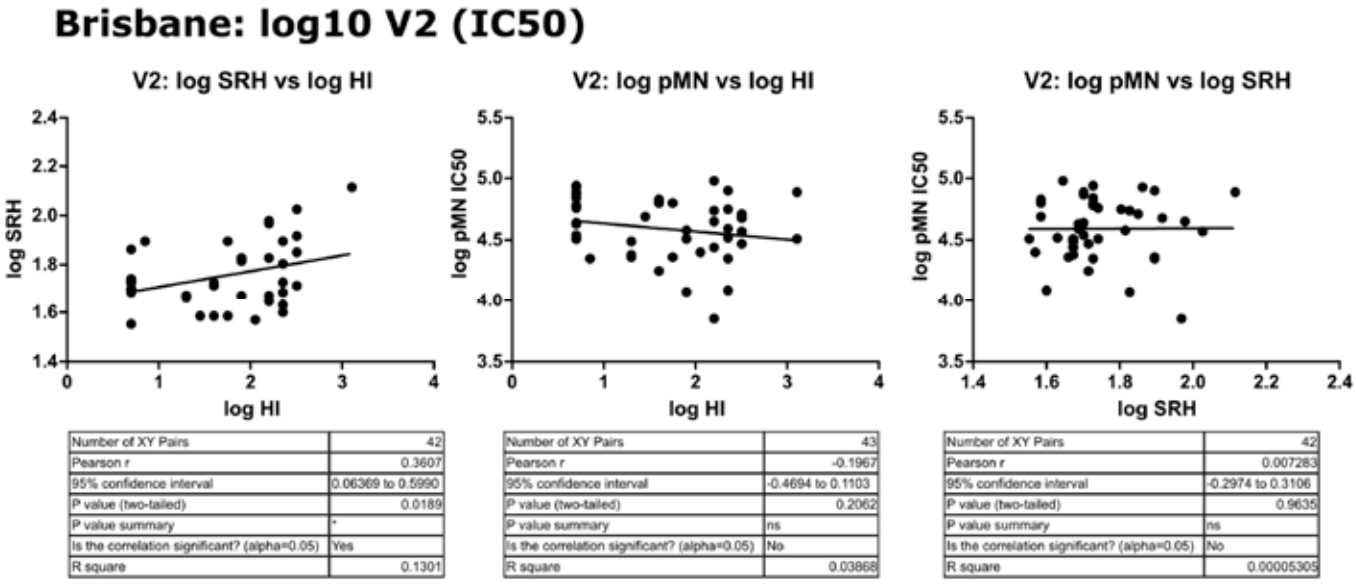


Figure 7. Correlation of SRH, HI and pMN (IC_{50}) V1 to V2 fold-change values assayed against B/Brisbane/60/2008 PV. Pearson's two-tailed analysis performed using GraphPad Prism. No correlation was observed between IC_{50} antibody titres and HI or SRH (Pearsons r = −0.07 and −0.08, respectively). Weak correlation was observed between SRH and HI (Pearsons r = 0.22), which was improved after removal of outlying HI value (Pearsons r = 0.60). $p > 0.05$ = ns, $p \leq 0.0001$ = ****.

3.4. Correlation of Data: B/Brisbane IC_{90} and Transformed Data

Upon transformation of the V2 data to a log10 scale, correlation was still not observed between pMN and SRH or HI despite normalization of scales, with a weak correlation between the latter two (r = 0.35, p = 0.0189). pMN IC_{90} data correlated weakly with HI and SRH (r = 0.37 and 0.45, p = 0.00188 and 0.0026, respectively). The correlation seen was reduced by a transformation of IC_{90} data and comparison on a log scale (r = 0.23 and 0.32, p = 0.132 and 0.0372, respectively) (see Figures 8–10).

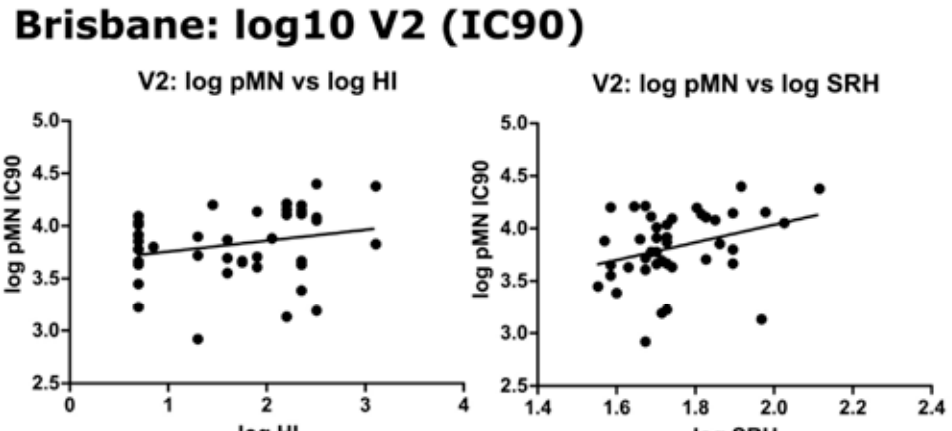


Number of XY Pairs	42
Pearson r	0.3607
95% confidence interval	0.06369 to 0.5990
P value (two-tailed)	0.0189
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R square	0.1301

Number of XY Pairs	43
Pearson r	-0.1967
95% confidence interval	-0.4694 to 0.1103
P value (two-tailed)	0.2062
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R square	0.03868

Number of XY Pairs	42
Pearson r	0.007283
95% confidence interval	-0.2974 to 0.3106
P value (two-tailed)	0.9635
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R square	0.00005305

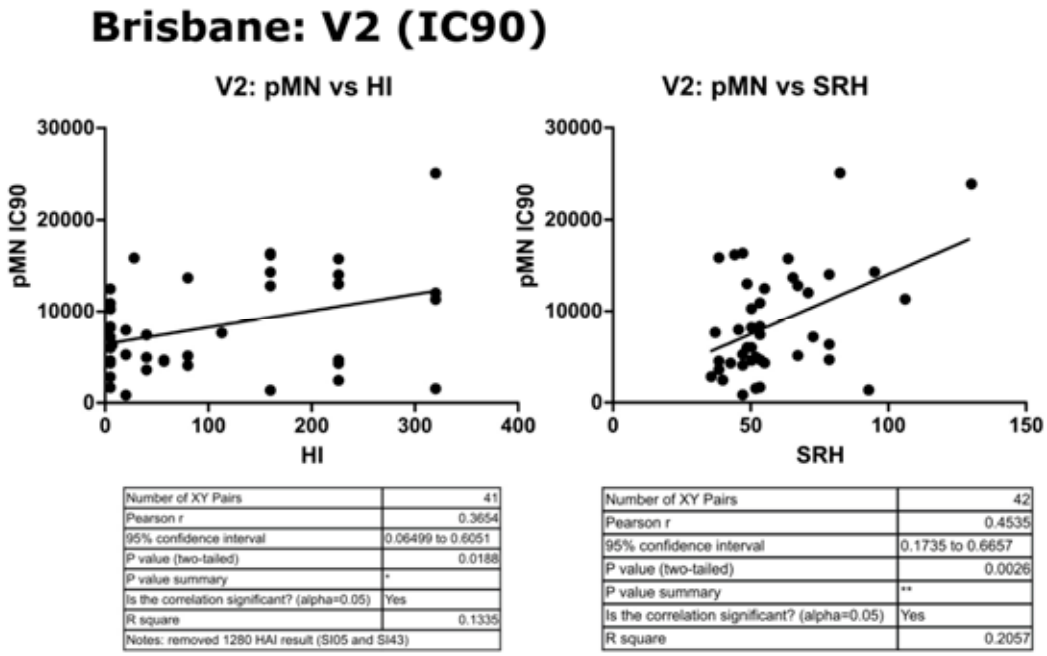
Figure 8. Correlation of transformed (log10) SRH, HI and pMN (IC_{50}) mean V2 values assayed against B/Brisbane/60/2008 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Weak correlation was observed between log SRH and HI values (Pearsons r = 0.36). No correlation was observed between log IC_{50} antibody titres and log HI or SRH values (Pearsons r = −0.196 and 0.007, respectively). $p > 0.05$ = ns, $p \leq 0.05$ = *.



Number of XY Pairs	43
Pearson r	0.2334
95% confidence interval	-0.07209 to 0.4988
P value (two-tailed)	0.132
P value summary	ns
Is the correlation significant? (alpha=0.05)	No
R square	0.05446

Number of XY Pairs	42
Pearson r	0.3226
95% confidence interval	0.02058 to 0.5706
P value (two-tailed)	0.0372
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R square	0.104

Figure 9. Correlation of transformed (log10) data for pMN (IC_{90}) with HI and SRH using mean V2 values for sera assayed against B/Brisbane/60/2008 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Weak correlation was observed between log IC_{90} antibody titres and log HI or SRH values (Pearsons r = 0.23 and 0.32, respectively). $p > 0.05$ = ns, $p \leq 0.05$ = *.



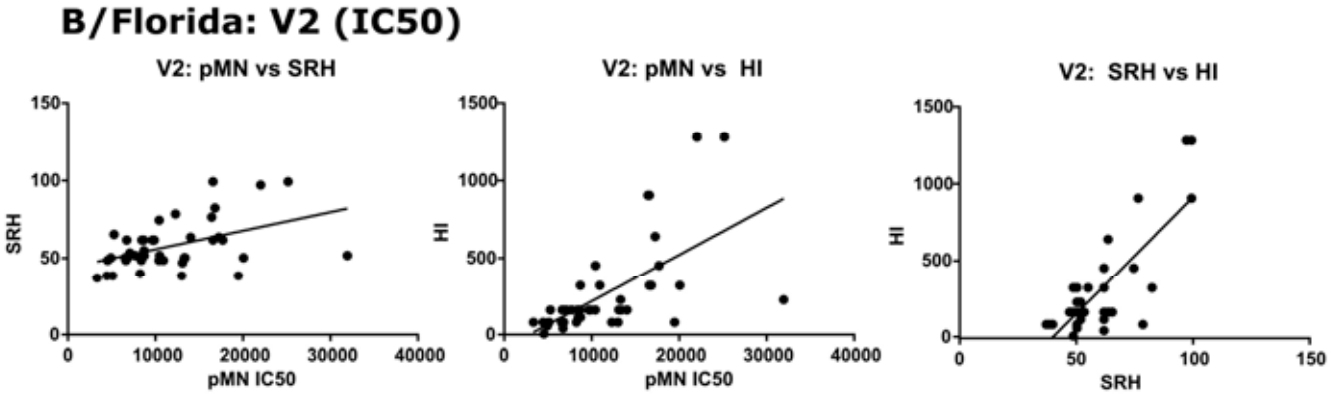
Number of XY Pairs	41
Pearson r	0.3654
95% confidence interval	0.06499 to 0.6051
P value (two-tailed)	0.0188
P value summary	*
Is the correlation significant? (alpha=0.05)	Yes
R square	0.1335
Notes: removed 1280 HAI result (SI05 and SI43)	

Number of XY Pairs	42
Pearson r	0.4535
95% confidence interval	0.1735 to 0.6657
P value (two-tailed)	0.0026
P value summary	**
Is the correlation significant? (alpha=0.05)	Yes
R square	0.2057

Figure 10. Correlation of pMN (IC_{90}) with HI and SRH using mean V2 values for sera assayed against B/Brisbane/60/2008 PV. Pearson's two-tailed analysis performed using prism Graph Pad. Weak correlation was observed between IC_{90} antibody titres and HI (Pearsons r = 0.36). IC_{90} values correlated well with SRH values (Pearsons r = 0.45). $p \leq 0.05$ = *, $p \leq 0.01$ = **.

3.5. Correlation of Data: B/Florida/4/2006 IC_{50}

In contrast to the results for B/Brisbane/60/2008, pMN data correlated well with SRH and HI, and V2 SRH and HI correlated strongly (r = 0.79, $p \leq 0.0001$) (see Figure 11). For the fold change data, pMN correlated with HI and SRH (r = 0.66 and 0.56, $p \leq 0.0001$). SRH and HI correlated strongly (r = 0.72, $p \leq 0.0001$) (see Figure 12). For V2 data, pMN correlated with SRH (r = 0.46, $p = 0.0023$) and HI (r = 0.61, $p \geq 0.0001$).



Number of XY Pairs	41
Pearson r	0.4624
95% confidence interval	0.1804 to 0.6742
P value (two-tailed)	0.0023
P value summary	**
Is the correlation significant? (alpha=0.05)	Yes
R square	0.2138

Number of XY Pairs	41
Pearson r	0.6108
95% confidence interval	0.3732 to 0.7732
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.373

Number of XY Pairs	41
Pearson r	0.7929
95% confidence interval	0.6417 to 0.8847
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.6287

Figure 11. Correlation of SRH, HI and pMN (IC_{50}) mean V2 values assayed against B/Florida/4/2006 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Weak correlation was observed between IC_{50} antibody titres and SRH (Pearsons r = 0.46). Strong correlation was observed between IC_{50} antibody titres and HI (Pearsons r = 0.61), as well as between SRH and HI (Pearsons r = 0.79) for the V2 data tested. $p \leq 0.01$ = **, $p \leq 0.0001$ = ****.

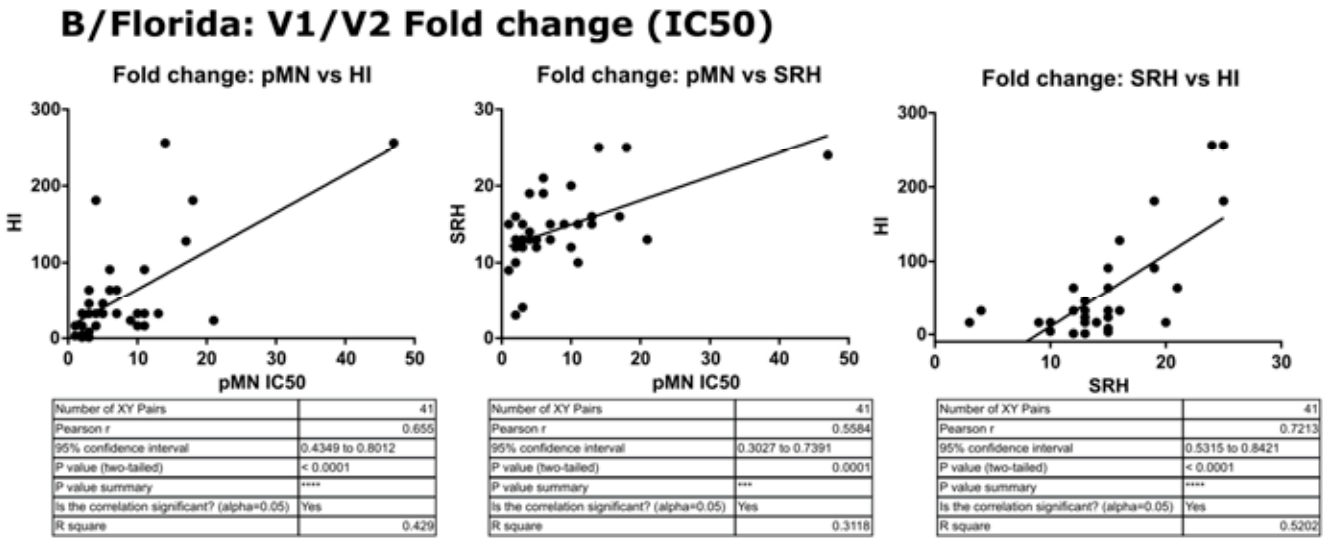


Figure 12. Correlation of SRH, HI and pMN (IC_{50}) V1 to V2 fold change values assayed against B/Florida/4/2006 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Strong correlation was seen between fold change in pMN and HI values between V1 and V2 antibody titres (IC_{50}), Pearsons r = 0.65. pMN and SRH V1/V2 fold changes correlated well, Pearsons r = 0.55 and strong correlation was observed for V1/V2 fold change for antibody titres of SRH and HI (Pearsons r = 0.72). $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

3.6. Correlation of Data: B/Florida/4/2006 IC_{90} and Transformed Data

Transformation to a log10 scale caused a slight reduction in the significance attributed to the correlation between pMN and SRH or HI (r = 0.57 or 0.30, p = 0.0002 or 0.0555), but correlation remained strong between all three assays. IC_{90} data correlated very well between pMN and SRH or HI (r = 0.85 and 0.65, $p \leq 0.0001$) and this was maintained when data were transformed to the log10 scale (r = 0.78 and 0.62, $p \leq 0.0001$) (see Figures 13–15).

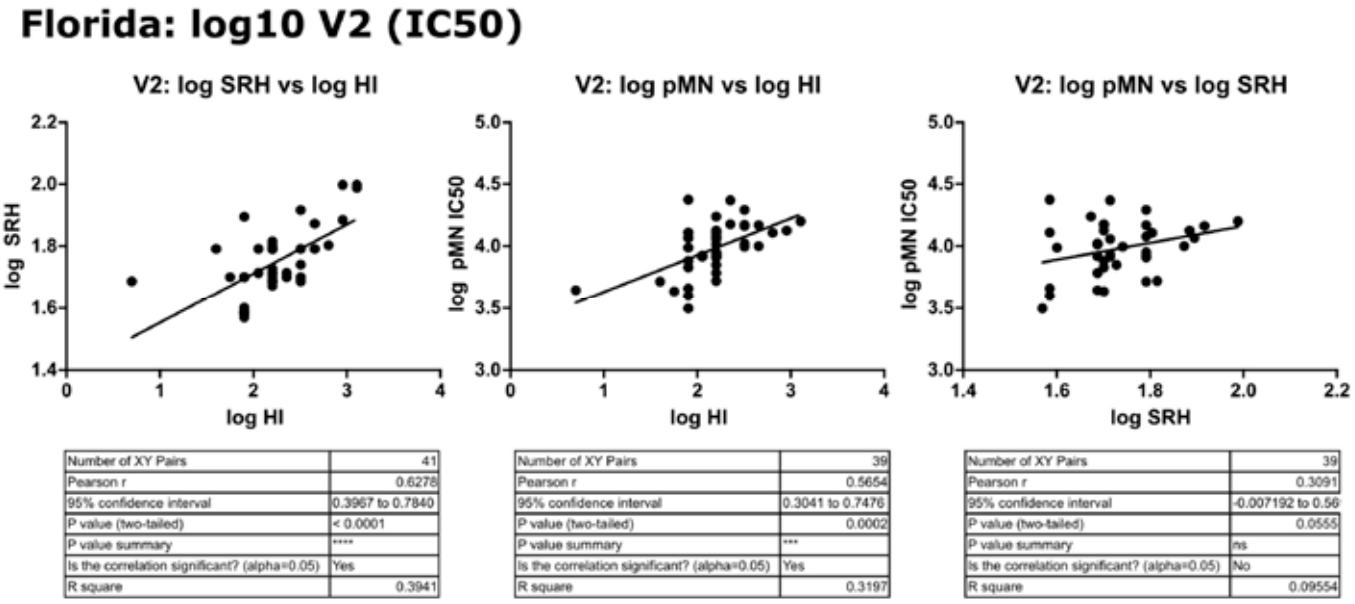
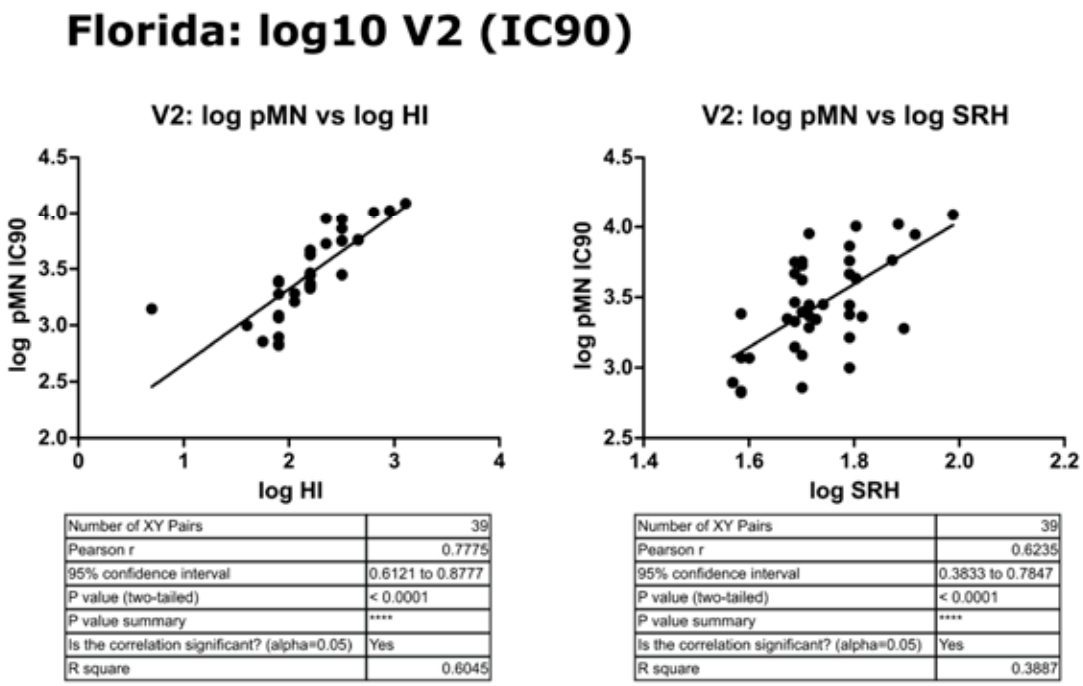


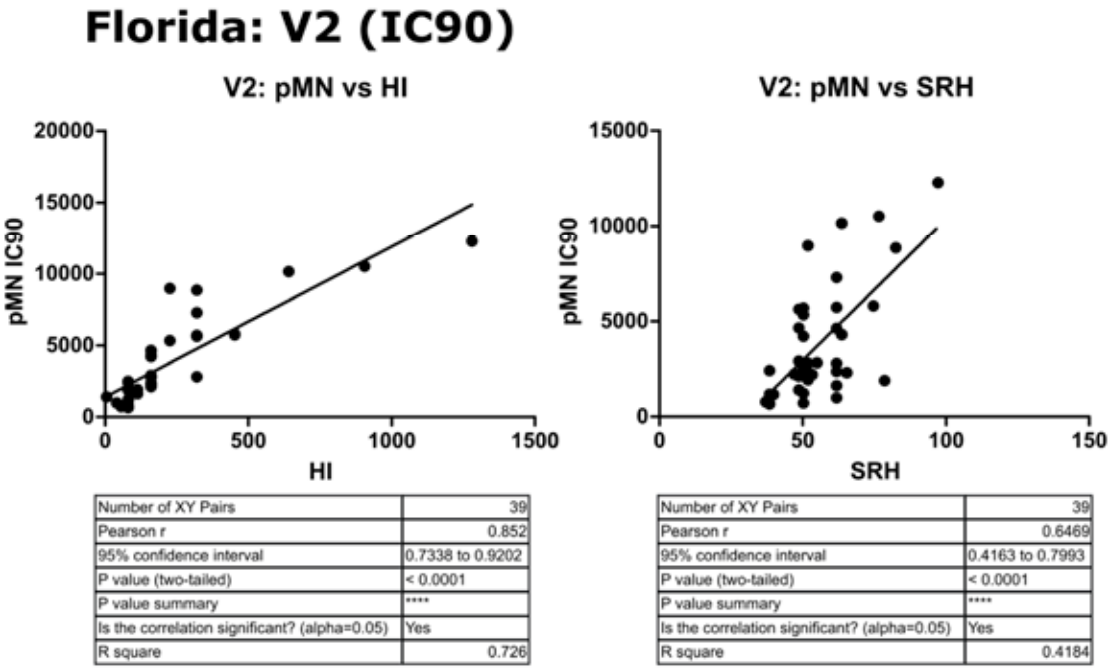
Figure 13. Correlation of transformed (log10) SRH, HI and pMN (IC_{50}) mean V2 values assayed against B/Florida/4/2006 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Strong correlation is observed between log SRH and HI (Pearsons r = 0.62. log pMN and log HI correlate well (Pearsons r = 0.56), and weak correlation was seen between log pMN and log SRH (Pearsons r = 0.30). $p > 0.05$ = ns, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.



Number of XY Pairs	39
Pearson r	0.7775
95% confidence interval	0.6121 to 0.8777
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.6045

Number of XY Pairs	39
Pearson r	0.6235
95% confidence interval	0.3833 to 0.7847
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.3887

Figure 14. Correlation of transformed (log10) data for pMN (IC_{90}) with SRH and HI mean V2 values, assayed against B/Florida/4/2006 PV. Pearson's two-tailed analysis performed using GraphPad Prism. Strong correlation was observed between log IC_{90} antibody titres and log HI or SRH values, with Pearsons r values of 0.77 and 0.62, respectively. $p \leq 0.0001$ = ****.



Number of XY Pairs	39
Pearson r	0.852
95% confidence interval	0.7338 to 0.9202
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.726

Number of XY Pairs	39
Pearson r	0.6469
95% confidence interval	0.4163 to 0.7993
P value (two-tailed)	< 0.0001
P value summary	****
Is the correlation significant? (alpha=0.05)	Yes
R square	0.4184

Figure 15. Correlation between pMN (IC_{90}) with SRH and HI mean V2 values, assayed against B/Florida/4/2006 PV. Pearson's two-tailed analysis performed using GraphPad prism. Strong correlation was observed between IC_{90} antibody titres and HI or SRH, with Pearsons r values of 0.85 and 0.64, respectively. $p \leq 0.0001$ = ****.

4. Discussion

Neutralisation of influenza B bearing PV with influenza B-specific mAbs was consistent, with the exception of B/Brisbane/60/2008 PV. Victoria-lineage-specific mAbs neutralised the type B/Victoria/2/1987 PV, while Yamagata-lineage-specific mAbs neutralised Yamagata

lineage strains (Figures 1–3). The cross-lineage-specific mAb 2F11 neutralised both lineages as well as the pre-lineage split strain B/Hong Kong/8/1973. Discordant correlation was observed between Victoria and Yamagata lineages, with the former correlating well between pMN, SRH and HI and the latter only correlating, weakly, between SRH and HI.

4.1. Neutralisation by Influenza B mAbs

While the majority of influenza B mAbs generated by the FDA neutralised the expected strains in the pMN assay, neutralisation was not seen for B/Brisbane/60/2008 by Victoria-specific mAbs, in contrast to data supporting the characterisation of these mAbs [40].

One possible explanation for this is N-linked glycosylation masking epitopes on the HA surface. The presence or absence of a glycosylated residue would dramatically affect results when neutralising with a mAb targetting one specific epitope. In this study, a WT B/Brisbane/60/2008 gene was used to produce PV for use in pMN, while B/Victoria/2/1987 was human-codon-optimised. HI and SRH made use of inactivated antigen produced in bacteria, whereas Verma and colleagues used a combination of ELISA using inactivated antigen and PV assays, with the latter using an egg-adapted strain of B/Brisbane/60/2008. Studies on egg-adaptation of influenza B viruses have characterised mutations within the 190 helix loop of the receptor-binding domain, which led to a significant change in antigenicity of the HA [55,56] due to the loss of an N-linked glycosylation site [57]. Despite the lack of neutralisation shown against B/Brisbane/60/2008 by the Victoria-specific mAbs in this study, polyclonal hyperimmune antisera produced against the same HA subtype was the most effective at neutralising it (Figure 4), suggesting that the HA itself is antigenically correct and that the problem lies in the specific epitopes targeted by 8E12 and 5A1. These mAbs were reported to bind to the amino acid residues 241 and 203, respectively, which are within or close to the 190 helix, which spans from residue 195 to 235 [40,58].

The fact that mAbs specific for the Victoria lineage also neutralised the pre-lineage split strain (Figure 1) is not surprising, as fewer structural differences have been reported between these than between the pre-lineage strain and the Yamagata lineage. The positions of escape mutations generated against the Victoria lineage-specific mAbs 8E12 and 5A1 presented in their characterisation were P241Q and K203R, respectively [40]. These are present within the 190 helix (RBD) of Victoria strains, which is very similar in amino acid composition to the 190 helix of the pre lineage split strain B/Hong Kong/8/1973 [58].

4.2. Correlation between SRH and HI

Overall, SRH and HI correlated strongly across both lineages, except for in V1 samples, which were predictably very low due to the lower sensitivity of these assays. As most of the V1 samples were negative in terms of HI and SRH data, they were clustered around the respective titres of 5 and 4 mm^2, which led to a lack of correlation through analysis. Fold change and V2 samples correlated for both Victoria and Yamagata lineages, indicating that increases in influenza B HA-specific antibodies are detected by both assays.

4.3. Correlation between pMN and SRH

pMN correlated strongly with SRH for the Yamagata lineage strain, with correlation observed for all data sets except for the negative or low titre samples that clustered to the lower detection limit of SRH at 4 mm^2. This suggests that pMN would be preferable for the evaluation of low-response samples, as its sensitivity offers an advantage over SRH. For B/Brisbane/60/2008, however, the only correlation observed was between IC_{90} values (transformed and raw data). Despite this, Pearson's r was still below 0.5 in each case.

4.4. Correlation between pMN and HI

As with pMN and SRH, a strong correlation is seen for the Yamagata strain (Figures 6–10) but not the Victoria strain (Figures 8–12); once again, B/Brisbane/60/2008 only poorly correlates when analysis is performed using IC_{90} values (Figure 10). B/Brisbane/60/2008

IC_{50} antibody titres do not correlate with SRH or HI when using either fold-change or V2 data, transformed or raw.

4.5. Limitations of This Study

Influenza B viruses have not undergone the ether treatment that could enhance their performance in the HI assay, despite reducing the assay specificity. The pseudotype-based assay represents a novel platform that is increasingly used by laboratories worldwide. However, each assay and antigen requires validation and optimization before this assay can compete with traditional HI or MN assays, which remain the gold standard for immunological outputs. In addition, as this was a single-cycle assay, the effect of neutralising or interfering antibodies on viral egress was not measured, contrary to live virus assays such as MN or PRNT, where an effect can be measured on viral egress. In our hands, the B/Brisbane/60/2008 PV did not perform as expected against characterised monoclonal antibodies, despite being strongly neutralised by polyclonal serum raised against the same antigen. This was a small panel of mAbs that would benefit from being expanded and evaluated on B/Brisbane/60/2008.

5. Conclusions

The results presented in this study, while discordant in regards to B/Brisbane/60/2008 PV, still represent the first comprehensive study correlating HI, SRH and pMN assays for influenza B serology. Lentiviral pseudotypes are becoming increasingly popular as a surrogate for live virus neutralisation assays—especially in current R&D using containment level 3 or 4 pathogens such as SARS-CoV-2 and Ebolavirus. The exquisite sensitivity of this assay allows the differentiation of serum samples that would otherwise be categorized as negative in the gold standard assays. This sensitivity can be a double-edged sword, as serum samples and reference antisera against the influenza B lentiviral pseudotypes reported in this study were strongly neutralising, leading to exceptionally high IC_{50} titers that may have been an overestimation of the serum potency. The nature of each assay described in this study is unique and detects antibodies that may interfere at different points in the virus life cycle. Neutralising antibodies are among the most important and often the goal for universal vaccine approaches—but are not explicitly detected by older assays including SRH and ELISA.

Many efforts have been made to standardise assays between platforms and laboratories to facilitate the comparison of different projects and data sets, such as the efforts by the Consortium for the Standardization of Influenza Seroepidemiology (CONSISE, https://consise.tghn.org/) and the establishment of reference standards and calibrants by the National Institute for Biological Standards and Control (NIBSC, nibsc.org). Until each pseudotyped virus has been fully validated and compared to traditional microneutralisation in a parallel and controlled setting, the gold standard assays will remain preferable for laboratories that are able to perform live virus assays.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-393X/9/2/100/s1, Figure S1: Titration of luciferase pseudotypes bearing the B/Brisbane/60/2008 haemagglutinin on HEK293T/17 cells. Quality control and production optimisation can be found as described previously.

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Development and Assessment of a Pooled Serum as Candidate Standard to Measure Influenza A Virus Group 1 Hemagglutinin Stalk-Reactive Antibodies

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Abstract: The stalk domain of the hemagglutinin has been identified as a target for induction of protective antibody responses due to its high degree of conservation among numerous influenza subtypes and strains. However, current assays to measure stalk-based immunity are not standardized. Hence, harmonization of assay readouts would help to compare experiments conducted in different laboratories and increase confidence in results. Here, serum samples from healthy individuals (n = 110) were screened using a chimeric cH6/1 hemagglutinin enzyme-linked immunosorbent assay (ELISA) that measures stalk-reactive antibodies. We identified samples with moderate to high IgG anti-stalk antibody levels. Likewise, screening of the samples using the mini-hemagglutinin (HA) headless construct #4900 and analysis of the correlation between the two assays confirmed the presence and specificity of anti-stalk antibodies. Additionally, samples were characterized by a cH6/1N5 virus-based neutralization assay, an antibody-dependent cell-mediated cytotoxicity (ADCC) assay, and competition ELISAs, using the stalk-reactive monoclonal antibodies KB2 (mouse) and CR9114 (human). A "pooled serum" (PS) consisting of a mixture of selected serum samples was generated. The PS exhibited high levels of stalk-reactive antibodies, had a cH6/1N5-based neutralization titer of 320, and contained high levels of stalk-specific antibodies with ADCC activity. The PS, along with blinded samples of varying anti-stalk antibody titers, was distributed to multiple collaborators worldwide in a pilot collaborative study. The samples were subjected to different assays available in the different laboratories, to measure either binding or functional properties of the stalk-reactive antibodies contained in the serum. Results from binding and neutralization assays were analyzed to determine whether use of the PS as a standard could lead to better agreement between laboratories. The work presented here points the way towards the development of a serum standard for antibodies to the HA stalk domain of phylogenetic group 1.

Keywords: influenza vaccine; serology; hemagglutinin; stalk; standardization

1. Introduction

As defined in the strategic plan from the National Institute of Allergy and Infectious Diseases [1], some of the key points to achieve the development of effective Universal Influenza Vaccines (UIV) include: the characterization of the immune responses elicited during influenza virus infection and vaccination; establishment of novel non-hemagglutination inhibition (HAI) correlates of protection; rational design of antigens with a wider breadth of protection; and implementation of these candidates in phase I-II clinical studies. Many current efforts towards the development of these novel types of vaccines rely on the induction of effective long-term antibody responses against conserved regions of the influenza virus glycoproteins [2].

The stalk domain of the hemagglutinin (HA) has been identified as a suitable target for universal influenza virus vaccines due to its unique properties. Contrary to the head domain, which is highly plastic [3], the stalk domain exhibits a high degree of conservation among numerous influenza virus subtypes and strains [4–7] but is immuno-subdominant [8,9]. As reviewed [10], anti-stalk antibodies act through diverse mechanisms including blocking the fusion of viral and cellular membranes [11–13], impeding the release of viral particles from infected cells [7,14], blocking the cleavage of the hemagglutinin [5], inducing complement activation [15] and triggering FcR-mediated effector functions, namely antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) [16,17]. Importantly, there is extensive evidence of the protective potential of anti-stalk antibodies in diverse animal models [6,7,14,16,18–21] and in humans [22–25]. Moreover, several vaccine candidates targeting this domain are in late pre-clinical, or early clinical stages of development [2,18,19,26,27].

Given the importance of qualitatively and quantitatively detecting antibody responses against the stalk in current research settings, and likely in future prophylactic scenarios for universal influenza virus vaccines, we initiated a collaborative project to investigate the possibility of developing an international

standard serum to measure group 1 HA stalk-reactive antibodies (group 1: H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18). As stated by the World Health Organization (WHO), 'reference standards are used as calibrators in assays' and define an internationally agreed, arbitrary unit that allows comparison of biological measurements worldwide [28]. There is a wide repertoire of WHO standards available, including 22 biological reference preparations in the "immunoglobulins and human sera" category and 83 biological reference preparations in the "vaccines/toxoids/toxins" category, with only two international standards available for influenza virus research [29]: (1) a standard, established in 2008, consisting of a pooled polyclonal serum obtained from individuals vaccinated with a clade 1 H5N1 virus (A/Vietnam/1194/2004) derived vaccine [30], and (2) the second International Standard for antibodies to pandemic H1N1 virus, consisting of pooled plasma from individuals who received a pandemic H1N1 split vaccine produced from the reassortant virus NYMC X-179A, derived from A/California/07/2009 [31]. Both standards were characterized by hemagglutination inhibition (HI) and virus neutralization (MN) assays. However, none of the available influenza antibody-standards are specific against the stalk of the HA. Therefore, the development of an international serum standard to measure stalk-reactive antibodies would have important implications worldwide, because it would contribute to the harmonization of assay read-outs, hence facilitating the comparison of experiments conducted in different laboratories and increasing confidence in results.

2. Materials and Methods

Cells, viruses, proteins and sera. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, MA, USA) and penicillin (100 U/mL)-streptomycin (100 µg/mL) solution (Gibco, NY, USA). Madin–Darby canine kidney cells (MDCK) were used for neutralization assays; MDCK cells expressing the protein cH6/1 (cH6/1-MDCK) (Chromikova et al., 2020), which contains the exotic avian HA head domain (H6) from an H6N1 virus (A/Mallard/Sweden/81/2002) and the stalk (H1) from an H1N1 virus (A/California/04/2009) were used for ADCC reporter assays; the sequence of the chimeric cH6/1 protein can be found in Supplementary Figure S1. ADCC Jurkat effector cells 187 FcγRIIIa V158 were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Paisley, UK) containing L-glutamine (Gibco, NY, USA), supplemented with 10% fetal bovine serum (FBS; HyClone, MA, USA), 100 µg/mL hygromycin (Invitrogen, CA, USA), 250 µg/mL antibiotic G-418 sulfate solution (Gibco, NY, USA), 1 mM sodium pyruvate (Gibco, NY, USA) and 0.1 mM minimal essential medium (MEM) of non-essential amino acids (Gibco, NY, USA). For enzyme-linked immunosorbent assays (ELISAs), the recombinant proteins cH6/1 (described above) and the mini-HA #4900 [18], which consists of a stabilized trimer of the stalk-domain from an H1N1 virus (A/Brisbane/59/2007), were used. To assess the neutralization capacity of the stalk-specific antibodies, a reassortant virus derived from an H1N1 virus (A/Puerto Rico/8/34) carrying the chimeric HA cH6/1 and the neuraminidase (NA) from an H12N5 virus (A/mallard/Sweden/86/2003) was used. The virus was grown in 8-day-old embryonated eggs (Charles River Laboratories, CT, USA) at 37 °C for 48 h. Human serum samples were obtained from a commercial vendor (110 samples). After testing, full units (volume~400 mL per donor) were purchased for the ten samples with highest reactivity to the HA stalk.

Direct ELISA. Antibodies in human serum were measured as described before [32]. In brief, ultra-high binding polystyrene 96-well plates (Immulon 4HBX; Thermo Scientific, PA, USA) were coated with 100 µL/well of recombinant protein in phosphate-buffered saline solution (PBS; pH 7.4; Gibco, NY, USA) at a concentration of 6 µg/mL for cH6/1 and 2 ug/mL for mini-HA #4900. Plates were incubated at 4 °C overnight, then washed 3 times with PBS containing 0.1% Tween 20 (PBS-T; Fisher Bioreagents, NJ, USA) using the plate washer system AquaMax 2000 (Molecular Devices, CA, USA). Blocking solution (220 µL/well) consisting of PBS-T, 3% goat serum (Gibco, OH, USA) and 0.5% non-fat dry milk (AmericanBio, MA, USA) was added to the plates, followed by incubation for 1–2 h. The serum was serially diluted (2-fold) from a 1:800 initial dilution for IgG and 1:100 for IgA. Samples were added to the plates (100 µL/well) and incubated at room temperature (RT) for

2 h. Plates were washed 3 times, and the specific secondary antibody (50 µL/well) was added at a 1:24,000 dilution. Goat Anti-Human IgG Fc specific horseradish peroxidase (HRP; Sigma, MO, USA) or Goat Anti-Human IgA α-chain specific HRP (Sigma, MO, USA) was used. After a 1 h incubation at RT, plates were washed 4 times and the substrate 3,3',5,5' tetramethylbenzidine (TMB, Bio-Rad, CA, USA) was added (100 µL/well). After a 30-min incubation, 50 µL/well of 4N H_2SO_4 solution (Thermo Fisher Scientific, MA, USA) was added. The optical density (OD) was measured at 450 nm using a Microplate Reader (Synergy H1, Biotek, VT, USA). Analysis was performed using Prism 7 software (GraphPad, San Diego, CA, USA), and values were reported as the area under the curve (AUC).

Competition ELISA. Coating using cH6/1 recombinant protein at 6 µg/mL and blocking were performed as for direct ELISAs. Plates were washed with PBS-T using the plate washer system AquaMax 2000 (Molecular Devices, CA, USA) as described above. Each lane on every 96-well plate was incubated for 2 h at RT with a specific serum sample to be tested at a 1:50 dilution in blocking solution (described above). Additionally, a non-competitor control plate was included, containing only blocking solution (described above). Two different setups for the competition ELISA assays were used according to the nature of the monoclonal antibody (mAb). For KB2 (mouse), 2-fold dilutions of the mAb (starting concentration of 0.08 µg/mL) were added (100 µL/well). Plates were incubated at RT for 2 h, washed 3 times, and incubated for 1 h with the secondary antibody Goat Anti-Mouse IgG (H&L) Antibody HRP (Rockland, PA, USA) at a 1:24,000 dilution (50 µL/well). MAb CR9114 (human) [7,33], was biotinylated using EZ-Link NHS-PEG4-Biotin (Thermo Scientific, IL, USA) and 2-fold dilutions of the mAb (starting concentration of 1 µg/mL) were added (100 µL/well) to every lane. Plates were incubated at RT for 2 h, washed 3 times, and incubated for 1 h with Pierce™ High Sensitivity Streptavidin-HRP (Thermo Scientific, IL, USA) at a 1:24,000 dilution (50 µL/well). Plates were washed 4 times, and substrate was added as described for direct ELISAs. Analysis was performed using Prism 7 software (GraphPad, San Diego, CA, USA), and values were reported as the percentage of competition between the antibodies contained in the serum samples and the mAbs.

Microneutralization assay (MN). Virus neutralization was assessed as previously described [34]. Briefly, MDCK cells maintained in DMEM (Gibco, NY, USA), supplemented with 10% FBS (HyClone, MA, USA) and Pen Strep (Gibco, NY, USA), were seeded in 96-well cell culture plates (Costar, DC, USA) and grown overnight at 37 °C with 5% CO_2 to reach an approximate confluence of 80–90%. Serum samples were treated with a receptor-destroying enzyme (RDE, Denka Seiken, Japan) according to the manufacturer's instructions and heat inactivated for 30 min at 56 °C. Serum samples were serially diluted (2-fold) from a 1:10 starting dilution in N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin-containing Ultra-MDCK medium (Lonza Bioscience, Belgium) and incubated for 1 h at room temperature with 100 times the 50% tissue culture infective dose ($TCID_{50}$) of cH6/1N5 virus, to allow binding of the antibodies to the virus. MDCK cell-medium was removed, cells were washed with PBS, 100 µL/well of the serum-virus mixture was added and plates were incubated at 37 °C. After an incubation period of 1 h, the serum-virus mixture was removed, cells were washed with PBS, and replaced with 100 µL/well of diluted serum at the previous concentration. Infection was let to proceed for 48 h. Supernatants were collected and used to perform hemagglutination assay using chicken red blood cells (concentration: 0.5%) as described before [35]. Data were analyzed using Prism 7 software (GraphPad, San Diego, CA, USA), and values were reported as microneutralization titers.

Antibody-Dependent Cellular Cytotoxicity assay (ADCC). Evaluation of effector functions of antibodies was performed using a commercial ADCC reporter kit according to the manufacturer's instructions (Promega, WI, USA). Briefly, cH6/1-MDCK cells were seeded in 96-well white flat bottom plates (Costar, ME, USA) at 3×10^4 cells/well and plates were incubated overnight at 37 °C with 5% CO_2. Serum samples were serially diluted (3-fold) starting from a 1:50 dilution in assay buffer consisting of RPMI 1640 medium supplemented with 0.5% low IgG FBS (Promega, WI, USA). Cell-growth medium was removed from cH6/1-MDCK cells and monolayers were washed with PBS (Gibco, NY, USA), followed by the addition of 25 µL/well of assay buffer and 25 µL/well of serum dilutions. Effector cells were thawed, washed and resuspended in assay buffer, and 7.5×10^4 cells/well were added to each

well in a volume of 25 µL. Plates were incubated at 37 °C with 5% CO_2 for 6 h. Bio-Glo Luciferase Assay Reagent (Promega, WI, USA) was added (75 µL/well) and luminescence was measured using a Microplate Reader (Synergy H1, Biotek, VT, USA). Data were analyzed using Prism 7 software (GraphPad, San Diego, CA, USA), and values were reported as AUC.

Pilot collaborative study and statistical analysis. Eight laboratories from six countries participated in the study (Table 1). A sample panel (Supplementary Table S1) consisting of a total of twelve blinded samples was shipped to participating laboratories; the panel consisted of a pooled serum (candidate standard) in duplicate (samples 6 and 10) and ten individual samples with varying levels of anti-stalk reactivity (high, intermediate and low), selected from the 110 tested serum samples; all samples were blinded to participating laboratories. Participants were requested to test all samples for anti-stalk antibodies using any assay(s) of their choosing, with a minimum of three independent tests per sample and laboratory, and to record their results on a results template, supplied by the National Institute for Biological Standards and Control (NIBSC). Results were submitted to NIBSC, where ED50s (the 50% effective dilution corresponding to a half-maximal assay response) were calculated for all binding assays (including those where only AUC was reported by the participant) by NIBSC's biostatisticians based on the submitted raw data where possible (one lab, for which independent calculation of ED50s was not possible, is indicated by an asterisk (*) in Supplementary Tables S4 and S5). Analysis was performed with a four-parameter logistic (sigmoid curve) model using the R package 'drc' [36] and a $\log_{10}$ transformation of the assay readout in all laboratories. In 1 case (laboratory 12), 1×10^6 was added to the assay readout value prior to log transformation and this was used as the assay response to calculate the sample ED50. Relative potencies were calculated by dividing the sample ED50 estimate by the corresponding assay ED50 estimate for the candidate standard sample 6. ED50 and potency estimates were combined as geometric means (GM) for each laboratory, and these laboratory means were used to calculate overall geometric means and overall median estimates for each sample. Variability between laboratories was expressed using geometric coefficients of variation (GCV = $[10^s - 1] \times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates). The extent of deviation of individual laboratory estimates from study consensus values was expressed as the fold-change in the laboratory GM from the overall study median estimate for that sample.

Table 1. Laboratories participating in pilot collaborative study.

Institution	Name	Country
Janssen Vaccines & Prevention	Boerries Brandenburg	The Netherlands
Centers for Disease Control and Prevention	Min Levine	United States
University of Bergen	Fan Zhou	Norway
Icahn School of Medicine at Mount Sinai	Adolfo García-Sastre & Teresa Aydillo-Gomez	United States
Vismederi Research Srl.	Alessandro Manenti	Italy
National Institutes of Health	Barney Graham	United States
University of Hong Kong	Sophie Valkenburg	China SAR
National Institute of Biological Standards & Control	Lethia Charles & Othmar Engelhardt	United Kingdom

3. Results

3.1. Generation of a Pooled Serum Containing High Levels of Stalk-Specific Antibodies

The production of a standard serum typically involves the collection of samples containing high levels of antibodies against the pathogen/molecule of interest. Several studies have demonstrated that some individuals possess higher levels of antibodies directed to the HA stalk of influenza viruses (likely induced by recent natural infection), and that these antibodies increase over time due to multiple exposures with influenza virus strains that are antigenically related [37,38]. Therefore, we decided to generate a standard serum containing high levels of stalk-specific antibodies by screening serum samples from healthy donors using a cH6/1 ELISA assay, which would allow us to detect antibodies directed specifically towards the stalk of group 1 HA influenza viruses (see workflow in Figure 1). Humans are naïve to the exotic avian H6 head domain, hence an undetectable amount of anti-head antibodies is present in human serum samples [39]. Anti-stalk antibodies measured using this chimeric protein have been shown to be an independent correlate of protection in humans [25]. Likewise, the cH6/1 construct, along with other chimeric constructs, has been used to assess stalk-specific antibodies in clinical trials for novel universal influenza vaccine candidates [27] (Nachbagauer et al., Nat Med. in press). Samples from a commercial vendor (n = 110) were screened for stalk-specific IgG titers (Figure 2A). The 10 samples with the highest reactivity were selected, and the full units from these donors were obtained (≈400 mL/sample). The full units were re-tested in the cH6/1 ELISA assay, to corroborate the presence of medium to high antibody titers against the stalk (Figure 2B). The 10 samples were mixed in equal proportions to generate a "pooled serum" that would comprise the model standard serum to be evaluated in this study, which exhibited high levels of IgG stalk-specific antibodies and relatively high levels of IgA stalk-specific antibodies (Figure 2C,D). Moreover, in order to characterize the properties of the antibodies contained in the serum samples, the samples were subjected to a panel of different assays that reflect the variety of tools currently available to detect group 1 stalk-reactive antibodies (Figure 1).

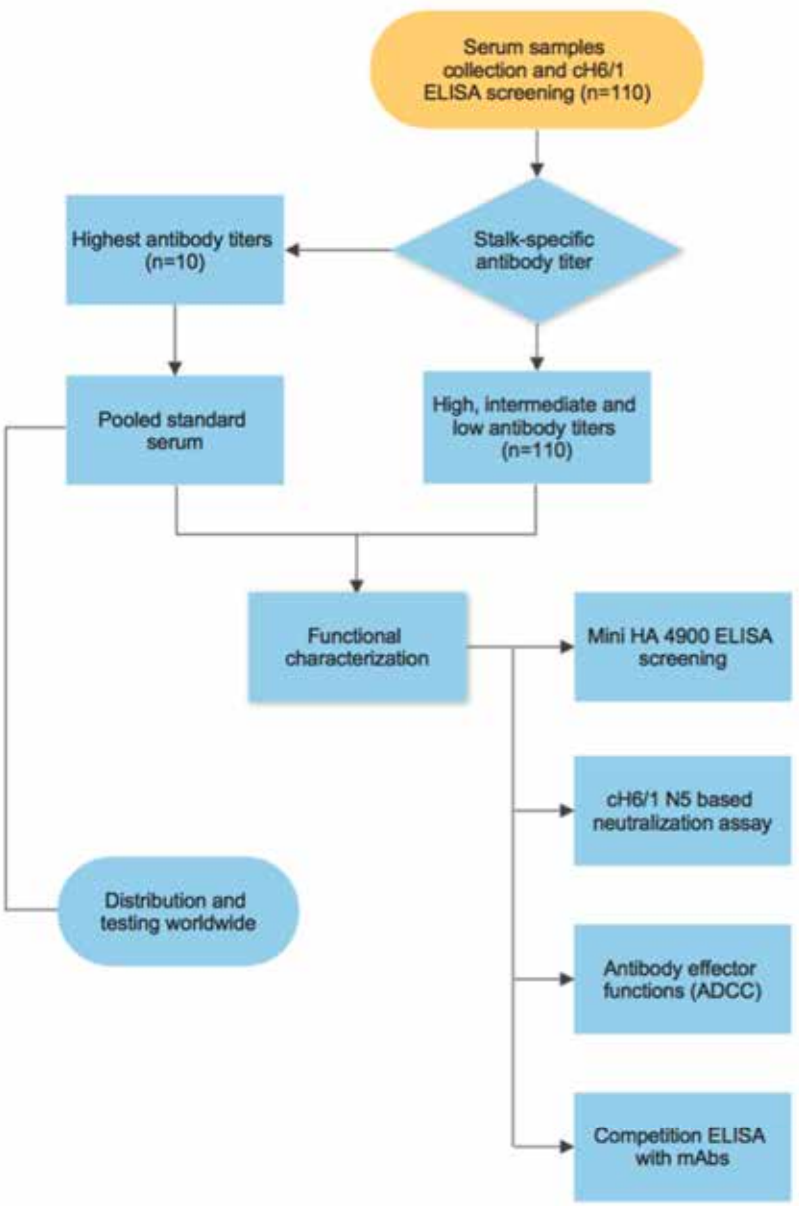


Figure 1. Workflow of the generation and characterization of a pooled serum as a candidate standard to measure influenza virus hemagglutinin stalk-reactive antibodies.

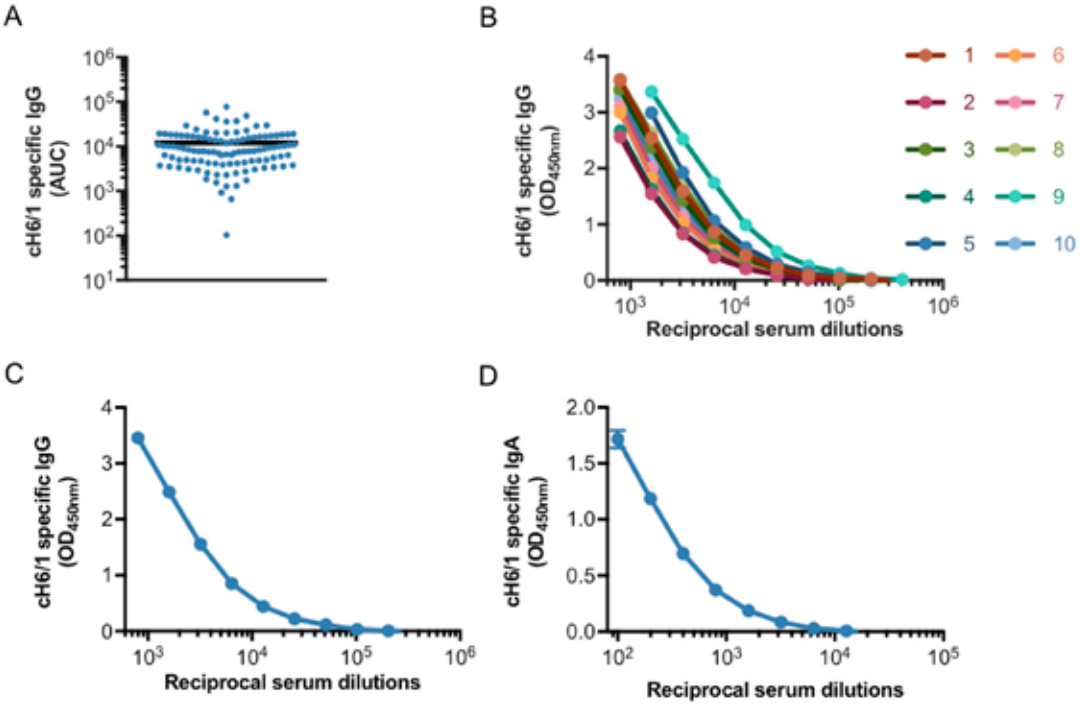


Figure 2. Testing and selection of human serum samples with high levels of stalk-specific antibodies. Samples of human sera were obtained from a commercial vendor ($n = 110$) and screened for stalk-specific IgG antibodies using a cH6/1-based enzyme-linked immunosorbent assay (ELISA) (**A**). The 10 samples with the highest IgG titers were selected, and the full units were obtained. The full units were re-tested for cH6/1-specific IgG titers (**B**). A pooled serum (PS) consisting of equal amounts of serum from each of the 10 full units was generated. The PS exhibited high levels of stalk-specific IgG (**C**) and IgA (**D**) antibodies. Dots in A represent individual values of the Area Under the Curve (AUC) from every serum sample, the arithmetic mean of all values is represented by a black horizontal line. Specific Optical Density (OD) for each of the serum dilutions is shown in (**A**–**C**).

3.2. Characterization of Stalk-Specific Antibodies Contained in Serum Samples

Different assays are used to measure and characterize stalk-reactive antibodies in basic and clinical research settings. These include: binding assays such as ELISA [27,32] and bio-layer interferometry (BLI) [40]; assays to assess the neutralizing capacity of antibodies, such as the MN assay [41,42] and plaque reduction assay [40]; and tests to characterize the effector functions of antibodies, including ADCP and ADCC [16,33]. Here, we characterized the properties of stalk-specific antibodies contained in serum samples from healthy donors, including the samples that comprise the candidate standard serum, by a panel of different assays. Measurement of stalk-specific IgG by ELISA against the trimeric headless construct #4900 (mini-HA), which is recognized by a panel of different monoclonal antibodies directed against the group 1 HA stalk [18], allowed us to detect samples with variable antibody levels (Figure 3A). Moreover, comparison between IgG antibody levels against the chimeric protein cH6/1 and those against the mini-HA #4900 showed a strong and significant correlation (Figure 3B, Pearson r^2: 0.7879; p (two-tailed): < 0.0001), which corroborates the presence and specificity of stalk-specific antibodies contained in the serum samples. To assess the neutralization capacity of the stalk-specific antibodies contained in the serum samples, we performed MN assays using the recombinant HA chimeric virus cH6/1N5, which allowed us to measure virus neutralization based on stalk-reactive antibodies. We found samples with variable levels of neutralization (Figure 3C), and a negligible correlation between the neutralization titers and the antibody levels measured in the cH6/1 ELISA was observed (Figure 3D, Pearson r^2: 0.05219; p (two-tailed): 0.059); this was as expected because only subsets of binding antibodies, which vary between individuals, display neutralizing activity. Moreover, despite binding, differences in the in vitro neutralization activity of stalk and head

antibodies are observed [43] and neutralization by stalk antibodies substantially depends on their effector functions such as ADCC activity [16], which play a role in vivo and are not detected in the in vitro microneutralization assay. Selected samples from low, intermediate and high responders in the cH6/1 ELISA were tested in an ADCC assay using a cell line stably expressing the chimeric HA cH6/1. Due to the low number of samples tested, correlation analysis could not be performed, however a positive association between the cH6/1 antibody titers and the ADCC activity was observed (Figure 3E), indicating that the stalk-reactive antibodies present in the serum samples possess effector functions, which may be important for in vivo protection [16]. Finally, in order to assess whether antibodies contained in the serum samples bind to some of the conserved epitopes in the stalk domain of the HA, we performed competition ELISAs using the widely characterized stalk-reactive monoclonal antibodies KB2 (mouse) and CR9114 (human) [7,33,44]. Using samples from high responders in the cH6/1 ELISA, we were able to detect a high percentage of competition (above 30% in most cases) between the monoclonal antibodies and the stalk-reactive antibodies contained in the serum samples (Figure 3F). In summary, these results confirmed the presence and specificity of stalk reactive antibodies in serum from healthy donors, using an array of different assays available for assessment of stalk-specific antibody responses.

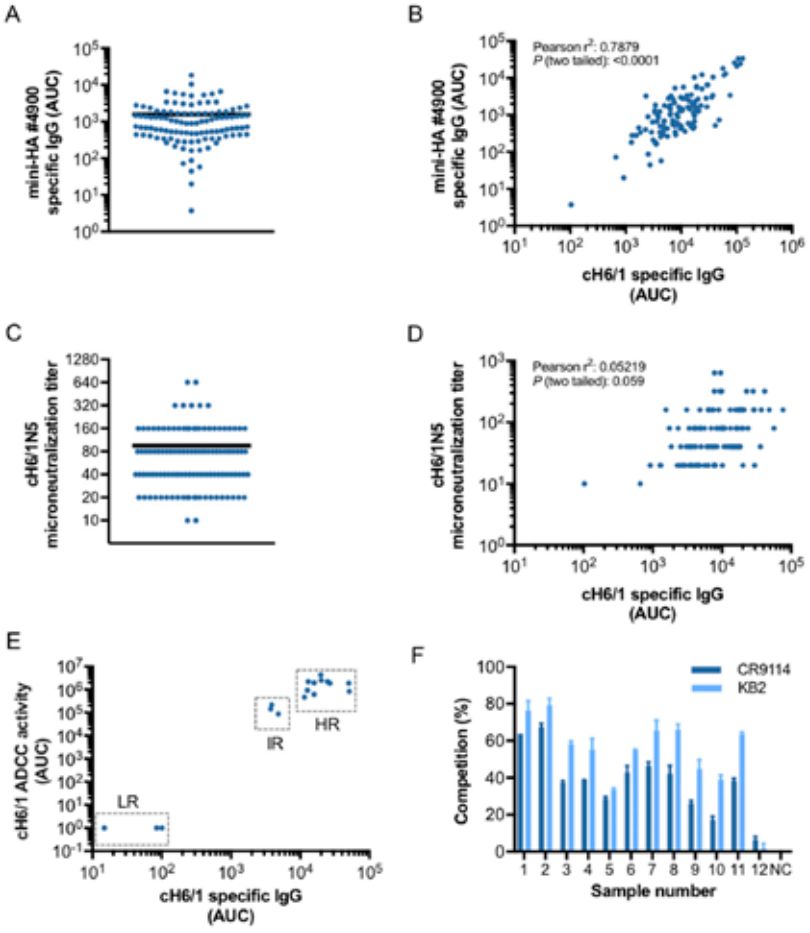


Figure 3. Characterization of stalk-specific antibodies contained in human serum samples. Human serum samples (n = 110) were screened for stalk-specific antibodies using a #4900 mini HA-based ELISA (**A**). The correlation between cH6/1-specific IgG and #4900 mini HA-specific IgG antibody levels is shown (**B**). Serum samples (n = 110) were subjected to a cH6/1N5-based neutralization assay. Microneutralization titers obtained are presented (**C**). The correlation between cH6/1-specific IgG and microneutralization titers is

shown (**D**). Samples from low, intermediate and high responders in the cH6/1 ELISA were tested in an ADCC commercial assay (n = 17). Association of the cH6/1-specific IgG levels with the effector functions of the antibodies measured in the ADCC assay is shown (**E**). Competition of the antibodies contained in the serum samples and the monoclonal antibodies KB2 (mouse) and CR9114 (human) was determined and presented as percentage of competition (**F**). Dots in (**A**,**B**,**D**,**E**), represent individual values of area under the curve (AUC) from every serum sample. The arithmetic mean of all values is represented by a black horizontal line. Pearson correlation coefficient (r^2) and p-value are shown in (**B**,**D**).

3.3. Testing of the Candidate Serum Standard in a Collaborative Study

The establishment of an international standard would require testing in multiple laboratories worldwide. Here, we conducted a pilot collaborative study to assess the potential of the candidate standard (pooled serum) to harmonize results from multiple assays from different laboratories. A sample panel consisting of the pooled serum and samples with varying levels of anti-stalk reactivity, selected from the 110 tested serum samples, was sent to the participating laboratories (Supplementary Figure S2, Table 1); all samples were blinded to participating laboratories. Participants were requested to test all samples for anti-stalk antibodies using any assay(s) of their choice, with a minimum of three independent tests per sample and laboratory. An array of different assays was used in the laboratories to assess binding and functional properties of stalk-reactive antibodies. Only binding assays and neutralization assays, for which results from at least two different laboratories were available, are reported here (Supplementary Table S2).

Participants were instructed to record their results on a specific template to allow a common analysis of all data at NIBSC. Not all participating laboratories returned data by the study deadline, while some laboratories supplied more than one dataset; laboratories were assigned random numbers, not related to the order of laboratories as shown in Table 1. Where the result from a single assay run caused the range of ED50 estimates to exceed eight-fold for a sample within a laboratory, the result was considered to be an outlier and was excluded from further analysis; the small number of cases where this occurred are indicated in Supplementary Table S3. Geometric mean ED50 estimates and geometric mean potency estimates relative to candidate standard sample 6 are shown in Figure 4 and Supplementary Tables S4 and S5. Samples 3 and 11 gave low relative potencies with GM < 0.05, below the limit of detection in some laboratories, and were therefore excluded from subsequent analysis. A reduction in between-laboratory %GCV when expressing titers as relative potencies was observed for all the other samples.

The extent of deviation from study consensus values for individual laboratories was assessed by calculating the fold-change of laboratory GM from the overall median estimate for each sample. This was calculated for both ED50 and relative potency estimates (Tables 2 and 3, Figure 5). Values closer to 1.0 indicate better agreement of a laboratory's result with the overall study median. Good inter-laboratory agreement (harmonization) when titers were normalized to the pooled serum (sample 6) was evident for labs 4, 7, 8, 9, 12a and 12b: 89–100% of potencies were within four-fold of the overall study sample median. In contrast, none of ED50 estimates for labs 9 and 12a were within this range without normalization (Figure 5A). Poorer agreement following normalization was observed for lab 11, while no change was evident for lab 10.

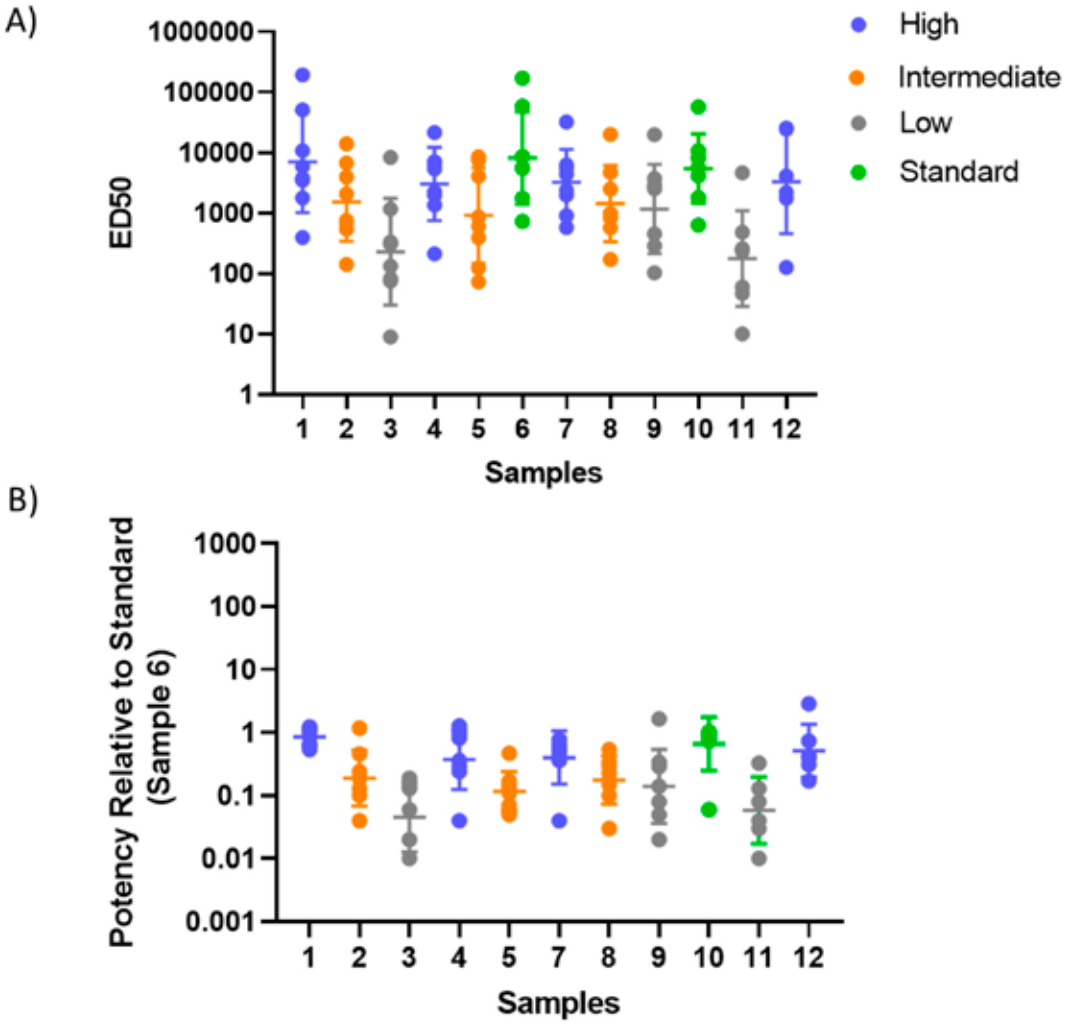


Figure 4. Geometric mean ED50 (50% effective dilution corresponding to a half-maximal assay response) and relative potency estimates. ED50 (**A**) and relative potency (**B**) estimates are shown for all samples (n = 11) and laboratories (n = 8). Each dot represents a geometric mean estimate for one sample and one laboratory. The same data are shown in Supplementary Tables S4 and S5 and Supplementary Figure S3.

Table 2. Inter-lab variability: Fold-change of laboratory geometric mean ED50 estimates from the overall study median ED50 estimate for each sample.

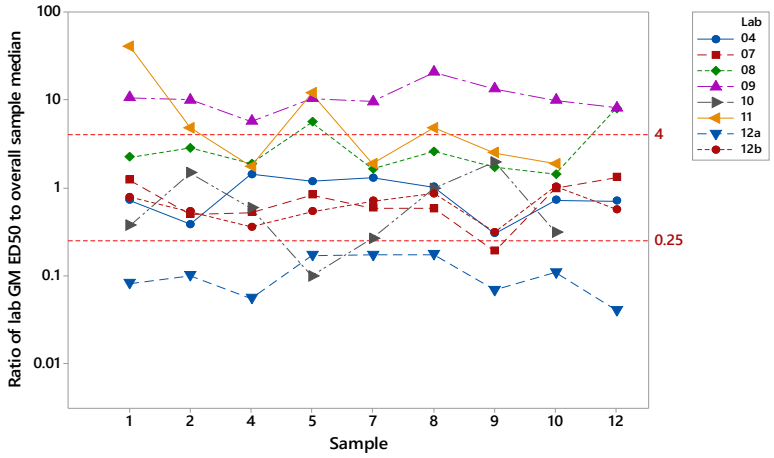
Sample	Laboratory							
	4	**7**	**8**	**9**	**10**	**11**	**12a**	**12b**
1	1.39	1.22	2.22	10.46	2.70	40.18	12.30	1.29
2	2.60	2.02	2.81	9.93	1.47	4.78	9.96	1.88
4	1.41	1.92	1.87	5.65	1.70	1.74	18.06	2.80
5	1.18	1.21	5.55	10.32	10.08	11.73	5.90	1.87
7	1.30	1.71	1.63	9.54	3.75	1.86	5.84	1.42
8	1.02	1.72	2.57	20.47	1.02	4.78	5.76	1.17
9	3.31	5.24	1.69	13.16	1.94	2.49	14.52	3.23
10	1.39	1.02	1.42	9.85	3.21	1.86	9.18	1.02
12	1.43	1.30	7.88	8.11	N/A	N/A	24.64	1.77
% < 2	**78%**	**78%**	44%	**0%**	**50%**	**34%**	**0%**	**78%**
% < 4	**100%**	**89%**	**78%**	0%	**88%**	**50%**	**0%**	**100%**
% < 8	**100%**	**100%**	**100%**	**11%**	**88%**	**75%**	**25%**	**100%**

: X < 2; : 2 < X < 4; : 4 < X < 8; : X > 8.

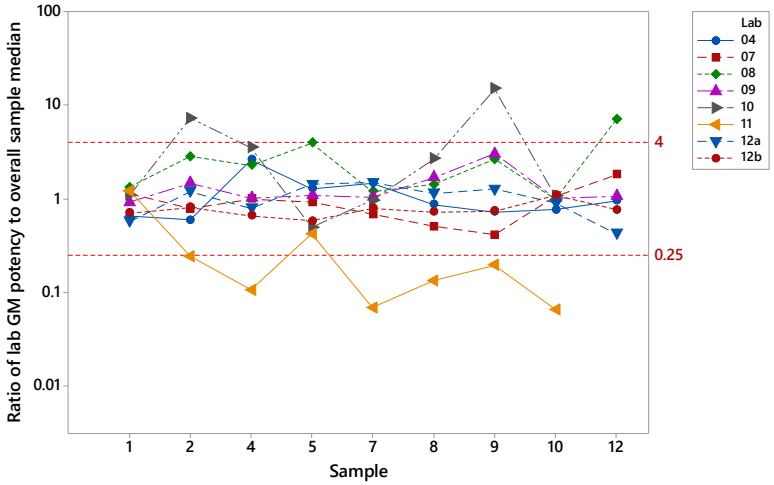
Table 3. Fold-change of laboratory geometric mean potency estimates from the overall median potency estimate for each sample.

Sample	Laboratory							
	4	**7**	**8**	**9**	**10**	**11**	**12a**	**12b**
1	1.53	1.14	1.32	1.09	1.08	1.21	1.75	1.43
2	1.70	1.28	2.82	1.47	7.23	4.13	1.19	* 1.23
4	2.61	1.01	2.26	1.01	3.50	9.39	1.26	* 1.52
5	1.28	1.09	3.94	1.08	2.03	2.38	1.42	* 1.73
7	1.46	1.48	1.20	1.04	1.04	14.49	1.50	* 1.27
8	1.16	1.98	1.42	1.68	2.67	7.48	1.14	1.39
9	1.39	2.44	2.63	3.02	14.83	5.12	1.27	* 1.36
10	1.31	1.07	1.01	1.01	1.07	15.34	1.12	1.08
12	1.06	1.80	6.96	1.06	N/A	N/A	2.36	* 1.32
% < 2	**89%**	**89%**	**44%**	**89%**	**34%**	**13%**	**89%**	**100%**
% < 4	**100%**	**100%**	**89%**	**100%**	**75%**	**25%**	**100%**	**100%**
% < 8	**100%**	**100%**	**100%**	**100%**	**88%**	**63%**	**100%**	**100%**

: X < 2; : 2 < X < 4; : 4 < X < 8; : X > 8. * max:min ratio reduced when potency expressed relative to sample 6.



(**A**)



(**B**)

Figure 5. Inter-laboratory variability in ED50 and relative potency estimates. Individual points show the ratio of laboratory geometric mean ED50 estimates (**A**) and relative potency estimates (**B**) to the study median ED50 estimate for that sample; range of 0.25–4 is shown to indicate points that are no more than 4-fold different from the study median.

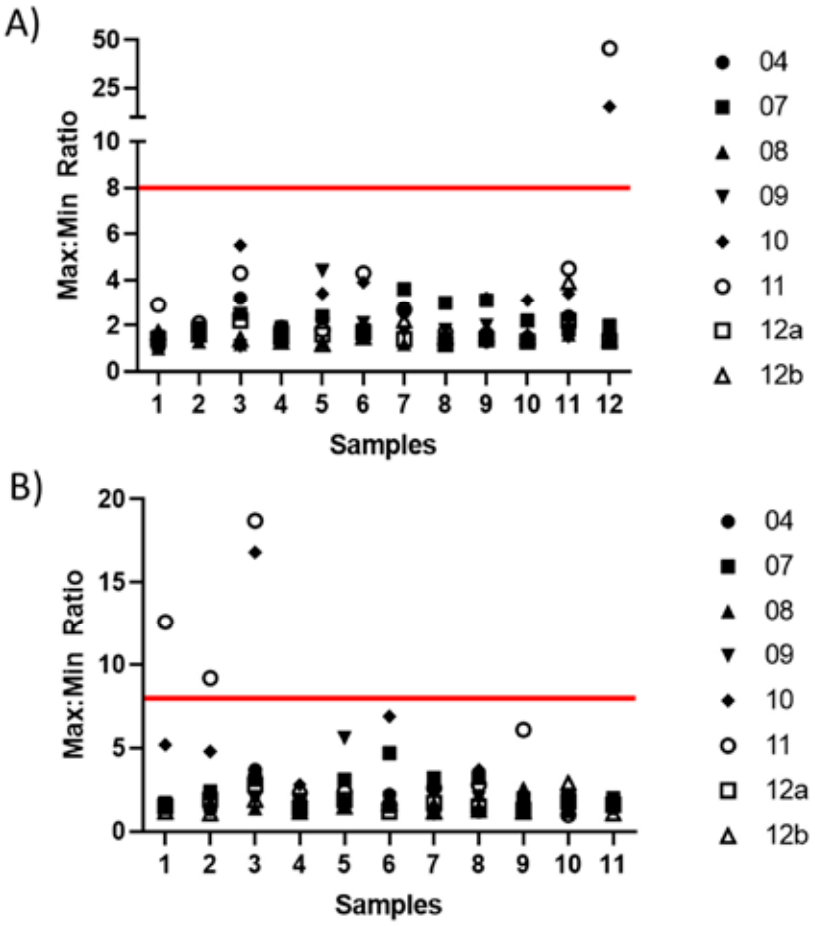


Figure 6. Intra-laboratory variability in ED50 and relative potency estimates. Individual points show the max:min ratio of laboratory ED50 estimates (**A**) and relative potency estimates (**B**) for each sample and laboratory; the red line marks a max:min ratio of 8-fold.

Only two laboratories returned data from virus neutralization assays, limiting the statistical analysis that could be performed. The differences in GM endpoint titer estimates and the GM relative potency estimates between the two labs for each sample are illustrated by the max:min ratios shown in Tables 4 and 5. The use of normalization relative to sample 6 reduced the max:min ratio for most samples (10 out of 11), indicating the potential for harmonization of results by the use of a standard.

Table 4. Geometric mean endpoint readout estimates—Virus Neutralization assays.

Sample	Laboratory		GM	Ratio	Max:Min Ratio
	04	10			
1	84.8	67.3	75.5	1.26	1.26
2	95.3	40	61.7	2.38	2.38
3	80	33.6	51.9	2.38	2.38
4	80	67.3	73.4	1.19	1.19
5	40	40	40	1.00	1.00
7	160	80	113.1	2.00	2.00
8	89.9	28.3	50.4	3.18	3.18
9	80	28.3	47.6	2.83	2.83
10	160	47.6	87.2	3.36	3.36
11	20	10	14.1	2.00	2.00
12	80	40	56.6	2.00	2.00

Shading shows ratios ≥ 2.00.

Table 5. Geometric mean potency estimates relative to Sample 6—Virus Neutralization assays.

Sample	Laboratory		GM	Ratio	Max:Min Ratio
	04	10			
1	0.53	0.59	0.56	0.89	* 1.11
2	0.60	0.35	0.46	1.68	* 1.71
3	0.50	0.30	0.39	1.68	* 1.67
4	0.50	0.59	0.55	0.84	* 1.18
5	0.25	0.35	0.30	0.71	1.40
7	1.00	0.71	0.84	1.41	* 1.41
8	0.56	0.25	0.37	2.25	* 2.24
9	0.50	0.25	0.35	2.00	* 2.00
10	1.00	0.42	0.65	2.38	* 2.38
11	0.13	0.08	0.10	1.59	* 1.63
12	0.50	0.35	0.42	1.41	* 1.43

Shading shows ratios ≥ 2.00. * max:min ratio reduced when potency expressed relative to sample 6.

4. Discussion

Multiple studies underline the importance of HA stalk-specific antibodies in the prevention and outcome of influenza virus infections [22–24]. Indeed, stalk-specific antibodies have been pointed out as independent correlates of protection [25]. Therefore, qualitatively and quantitatively measuring these particular types of antibodies and comparison of laboratory results among different research groups are essential. Here, we generated, characterized, and tested a candidate serum standard for stalk-reactive antibodies in humans in an international pilot collaborative study. The candidate standard exhibited high levels of stalk-reactive antibodies, a high neutralization titer, and displayed strong antibody-effector functions such as high levels of ADCC activity.

Results obtained from the international pilot study support the concept that implementation of a standard would improve the harmonization of results from different laboratories. Normalization of results to the standard improved inter-laboratory variability of stalk-specific antibody levels. The generation of a standard based on human sera ensures that the matrix of the reagent is compatible with analysis of stalk-reactive antibodies in human serum samples, providing commutability with samples of clinical importance, such as samples from clinical trials of prophylactic approaches and in diagnostic settings. Moreover, antibodies in human serum samples stored at −20 °C are well preserved for prolonged periods of time [45]; therefore, we anticipate that a future international standard, generated analogously to the one described here, and stored at low temperature after lyophilization, will be stable [46]. Even though normalization to the candidate standard did not improve agreement to the overall median for all laboratories or all samples, this observation is not unusual [47,48]. Reduced harmonization may have been due to multiple causes, including inexperience of a laboratory with a particular assay, variability of reagents used, or systematic differences between tests of the standard and the samples. Moreover, testing results exhibited similar patterns of inter- and intra- laboratory variability as seen with other standards [46,49].

In summary, these results suggest that the use of a standard has the potential to facilitate the comparison of experiments to measure stalk-specific antibodies conducted in different laboratories and to increase confidence in results. We therefore conclude that the generation of an international standard, based on the results of this Phase 1 project, and using the same pool of high-titer samples that was tested in this study, is worthwhile, and will be of use in the development and assessment of vaccine candidates targeting the HA stalk domain.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-393X/8/4/666/s1, Figure S1: Nucleotide (open reading frame) and amino acid sequences corresponding to the chimeric protein cH6/1 used in this study. Figure S2: The pooled serum displays high levels of stalk-specific antibodies with functional properties; Figure S3: Individual laboratory geometric mean potencies relative to candidate standard sample 6; Table S1: Sample Panel; Table S2: Assays performed in different laboratories; Table S3: Sample ED50s excluded from analysis; Table S4: Geometric mean ED50 estimates; Table S5: Geometric mean potency estimates relative to sample 6; Table S6: Intra-lab variability: Ratios of the maximum and minimum ED50s for each sample

in each laboratory; Table S7: Intra-lab variability: Ratios of the maximum and minimum potencies for each sample in each laboratory.

Author Contributions: Conceptualization, O.G.E. and F.K.; Formal analysis, P.R. and E.A.; Funding acquisition, O.G.E. and F.K.; Investigation, J.M.C., T.H., L.C. (Lethia Charles)., R.N., M.A.B., S.S., L.C. (Lynda Coughlan), T.A., B.B., K.K., M.Z.L., A.M., A.B.M., L.M., S.R.N., R.A.P.M.P., S.A.V. and F.Z.; Methodology, J.M.C. and F.K.; Project administration, J.M.C., J.U.M., O.G.E. and F.K.; Resources, F.K.; Supervision, A.G.-S., A.B.M., E.M., S.A.V., O.G.E. and F.K.; Writing—original draft, J.M.C., J.U.M., P.R. and O.G.E.; Writing—review and editing, J.M.C., J.U.M., T.H., P.R., E.A., L.C. (Lethia Charles), R.N., T.A., B.B., M.Z.L., A.M., A.B.M., E.M., L.M., S.R.N., N.C.S., S.A.V., F.Z., O.G.E. and F.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The Icahn School of Medicine at Mount Sinai (ISMMS) has issued patents and filed patent applications covering the use of chimeric hemagglutinin antigens as vaccines. RN, AG-S, and FK are named as inventors on these patents and applications. The ISMMS and the inventors have received payments as consideration for these rights. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The findings and conclusions presented here are the authors' and do not necessarily reflect positions or policies of the Bill & Melinda Gates Foundation.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC or funding agencies.

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Serologically-Based Evaluation of Cross-Protection Antibody Responses among Different A(H1N1) Influenza Strains

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Abstract: After the influenza H1N1 pandemic of 2009, the seasonal A/Brisbane/59/2007 strain was replaced by the A/California/07/2009 strain for the influenza virus vaccine composition. After several seasons with no indications on the occurrence of antigenic drift, A/Michigan/45/2015 was chosen as the H1N1 vaccine strain for the 2017/2018 season. Since the immune response to influenza is shaped by the history of exposure to antigenically similar strains, the potential cross-protection between seasonal human influenza vaccine strains and the emerging pandemic strains was investigated. Human serum samples were tested by hemagglutination inhibition and single radial hemolysis assays against A/Brisbane/59/2007, A/California/07/2009, and A/Michigan/45/2015 strains. Strong cross-reactions between A/California/07/2009 and A/Michigan/45/2015 strains were observed in 2009/2010, most likely induced by the start of the 2009 pandemic, and the subsequent post-pandemic seasons from 2010/2011 onward when A/California/07/2009 became the predominant strain. In the 2014/2015 season, population immunity against A/California/07/2009 and A/Michigan/45/2015 strains increased again, associated with strong cross-reactions. Whereas hemagglutination inhibition assay has a higher sensitivity for detection of new seasonal drift, the single radial hemolysis assay is an excellent tool for determining the presence of pre-existing immunity, allowing a potential prediction on the booster potential of influenza vaccines against newly emerging drifted strains.

Keywords: influenza virus; H1N1; immunity; antigenic drift

1. Introduction

The immune response to influenza is shaped by the individual history of exposure to related antigens. Individuals who have been exposed to a greater number of influenza strains, through natural infection or vaccination, likely harbor pre-existing memory responses that cross-react with vaccine strains.

A new A/H1N1 influenza virus began circulating in humans in the spring of 2009, causing the first pandemic of the 21st century. The new virus (A/California/07/2009 H1N1pdm09) was the result of a triple reassortment from human, swine, and Eurasian avian influenza viruses and affected mostly children and young adults [1,2]. This could be due to the similarity of the H1N1 pdm09 strain to the viruses circulating in humans between the 1930s and 1950s, suggesting that adults, in particular those over 60 years old, could have some cross-reactive antibody responses against the pandemic virus [3–6], as proven by several studies [3,4,7–11].

The recommendation for the H1N1 component for the influenza vaccines changed from A/New Caledonia/20/1999-like strain in seasons 2005/2006 and 2006/2007 to A/Solomon Island/03/2005-like strain in season 2007/2008 and subsequently to A/Brisbane/59/2007-like strain in the 2008/2009 and 2009/2010 seasons. However, in 2009, A/California/07/2009 emerged as a pandemic strain and replaced A/Brisbane/59/2007 as the H1N1 circulating strain. Consequently, the World Health Organization (WHO) provided a vaccine recommendation for A/California/07/2009 as the seasonal H1N1 vaccine strain after the end of the pandemic. In 2017/2018 season, the WHO recommendation changed to A/Michigan/45/2015-like (Table 1) [12]. Here, we refer to the recommendations until 2009/2010 as "pre-pandemic," 2009 and 2010 as "pandemic," and from season 2010/2011 onward as "post-pandemic."

Table 1. Influenza A/H1N1 strain egg-based vaccine composition Northern Hemisphere (NH) for the 2005/2006–2020/2021 seasons. Shown in bold the first time the strains considered in this study were included in WHO vaccine recommendation.

NH Season	A/H1N1 Strain
2005/2006	A/New Caledonia/20/99-like
2006/2007	A/New Caledonia/20/99-like
2007/2008	A/Solomon Island/3/2005-like
2008/2009	**A/Brisbane/59/2007-like**
2009/2010	A/Brisbane/59/2007-like
2010/2011	**A/California/7/2009-like**
2011/2012	A/California/7/2009-like
2012/2013	A/California/7/2009-like
2013/2014	A/California/7/2009-like
2014/2015	A/California/7/2009-like
2015/2016	A/California/7/2009-like
2016/2017	A/California/7/2009-like
2017/2018	**A/Michigan/45/2015-like**
2018/2019	A/Michigan/45/2015-like
2019/2020	A/Brisbane/02/2018-like
2020/2021	A/Guangdong-Maonan/SWL1536/2019-like

Hemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins of influenza viruses, both recognizing sialic acid (SA). HA binds to SA on the host cells, allowing virus entry, whereas NA has enzymatic activity removing SA, facilitating the release of progeny virus [13]. HA is the major target of humoral immune response and rapid antigenic variation due to the accumulation of mutations results in antigenic drift. In addition, periodic reassortment between antigenically distinct influenza viruses can lead to antigenic shift and the emergence of pandemic strains [14]. The HA molecule comprises a membrane-distal domain (globular head, HA1) and a membrane-proximal domain (stalk, HA2). Most antibody responses against HA are strain-specific, targeting HA1. Alternatively, HA2 is highly conserved compared with the globular head, making it a suitable target for vaccine development to induce broad and protective immune responses [15].

The immunological response to influenza vaccine and natural infection is mainly evaluated by two serological techniques, hemagglutination inhibition (HI) and single radial hemolysis (SRH), both of which are still the most widely used and officially recognized serological assays by regulatory authorities. In recent years, the importance of neutralization assays for serological evaluations has

increased and the analysis of neuraminidase-specific antibodies has also become a topic of increasing interest [16]. However, the HI assay is still considered the gold standard for antigenic and serological analysis [17]; it still widely serves as a correlate of protection for influenza vaccines in detecting antibodies able to inhibit the interaction between red blood cells (RBCs) and the globular head of HA, i.e., blocking of receptor-binding activities and subsequent inhibition of infection events. The detected antibodies seem to be strain-specific and not protective against mismatching influenza strains [16].

Alternatively, the SRH assay measures complement fixation antibodies, mainly immunoglobulin (Ig) G (IgG1 and IgG3), not only against the surface glycoproteins HA and NA, but also against internal antigens, leading to a potential lack of specificity to HA antibodies [18,19].

For decades, an HI titer ≥40 and a SRH hemolysis area of 25 mm^2 or greater have been acknowledged as correlates of protection and were considered the protective threshold level beyond which the probability of contracting influenza infection was reduced by 50% or more. Since February 2017, the revised guidelines on influenza vaccines by the European Medicine Agency (EMA) have withdrawn the traditional concept of HI and SRH as correlates of protection [20], in contrast to other representative regulatory authorities such as United States Food and Drug Administration (U.S. FDA). However, the debate on HI and/or SRH representing a correlate of protection (CoP) or at least a surrogate of protection (SoP) is still ongoing [16].

The aim of this study was to investigate the specificity of the HI and SRH assays with respect to influenza virus antigenic drift and shift variants and potential cross-reactivity between seasonal human influenza H1N1 vaccine strains and the emerging pandemic H1N1 strain of swine origin. Therefore, A/Brisbane/59/2007 was chosen as the last recommended H1N1 strain of the pre-pandemic seasons until 2008/2009, representing the H1N1 viruses circulating in the human population since then. The pandemic strain A/California/07/2009 was chosen as the representative strain for the 2009/2010 pandemic season and the post-pandemic seasons from 2010/2011 onward, including A/Michigan/45/2015 as a drift variant of A/California/07/2009, recommended by the WHO as the H1N1 vaccine strain of the 2017/2018 season.

2. Materials and Methods

2.1. Virus Antigens

The infectious influenza A/H1N1 viruses used for serological assays were seasonal influenza strains obtained from the National Institute for Biological Standards and Control (NIBSC): A/Brisbane/59/2007 (H1N1, 09/276), A/Michigan/45/2015 (H1N1, 16/354), and A/California/07/2009 (H1N1, 09/216). All viruses used were egg-grown.

2.2. Serum Samples

Human serum samples were obtained from the Sera Bank of the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy. The samples were anonymously collected and stored in compliance with Italian ethics law.

Serum samples were collected from the general population in Italy between January 2005 and August 2017. Out of 1000 samples available for every single season, 100 samples were randomly selected for each season included in this study (2005/2006, 2008/2009, 2009/2010, 2010/2011, 2013/2014, 2014/2015, 2015/2016, and 2016/2017) balanced between two age groups, 18–65 years old (younger adults, $n = 50$) and >65 years old (elderly adults, $n = 50$).

2.3. Hemagglutination Inhibition Assay

All serum samples were pre-treated with receptor destroying enzyme (RDE) (ratio 1:5) from Vibrio Cholerae (Sigma Aldrich, St. Louis, MO, USA) for 18 h at 37 °C in a water bath and then heat inactivated for 1 h at 56 °C in a water bath with 8% sodium citrate (ratio 1:4).

Turkey RBCs (TRBCs) were centrifuged two times, washed with 0.9% saline solution, and adjusted to a final dilution of 0.35%.

From an initial dilution of 1:10, serum samples were 2-fold diluted in duplicate with 0.9% saline solution in a 96-well plate. Twenty-five microliters of standardized viral antigen were added to each well and the mixture was incubated at room temperature for one hour. TRBCs were added and, after one hour of incubation at room temperature, the plates were evaluated for the presence of agglutination inhibition.

The antibody titer is expressed as the reciprocal of the highest serum dilution showing complete inhibition of agglutination. Since the starting dilution was 1:10, the lower limit of detection (LoD) for the antibody titer is 10. When the titer was under the detectable threshold, the results were conventionally expressed as 5 for calculation purposes (half the lowest detection threshold).

2.4. Single Radial Hemolysis Assay

Serum samples were heat inactivated at 56 °C for 30 min in a water bath.

Fresh TRBCs were centrifuged and washed with phosphate buffer saline (PBS) twice. Diluted virus antigen was added to the TRBCs suspension at a concentration of 2000 hemagglutinin units (HAU) per mL. To allow the adsorption of viral antigen to the TRBCs, the suspension was incubated at 4 °C for 20 min. A solution of 2.5 mM chromium chloride ($CrCl_3$) was added to the previous suspension and incubated at room temperature for 10 min to increase the binding affinity between the TRBCs and the viral antigen. The suspension was carefully mixed and centrifuged. The supernatant was removed and PBS was added to the pellet, which was then carefully re-suspended. A stock solution of 1.5% agarose in PBS containing 0.1% sodium azide and 0.5% low gelling agarose was prepared. The agarose stock solution was kept at 45 °C in a water bath.

Each SRH plate consisted of TRBCs—viral antigen suspension and guinea pig complement in the agarose mixture. The final suspension was homogenized by shaking and spread onto each plate, incubated at room temperature for 30 min and then stored at 4 °C for 30–90 min to set the agarose. Holes were punctured in each plate with a calibrated punch and 6 µL of serum samples and controls added into each hole. The plates were stored in a humid box and incubated at 4 °C for 16–18 h in the dark. After overnight incubation, the plates were further incubated in a water bath at 37 °C for 90 min and then the diameters of hemolysis areas read in millimeters with a calibrating viewer [19].

2.5. Statistical Analysis

All statistical analyses were performed using Microsoft R-Open version 3.5.0 (R Core Team, 2018, city, country). R is a language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria). For the purpose of this study, only samples with an HI titer ≥ 40 and SRH hemolysis area ≥ 25 mm^2 were considered positive.

The results from the HI and SRH assays are reported as a proportion of positive samples along different seasons' antigens, all ages, and age groups (18–65 years old and >65 years old) separately. Corresponding 95% confidence intervals (CIs) were calculated by the Wilson method using the DescTools R-package. Venn diagrams reporting proportions of cross-protection were prepared using the VennDiagram R-package. A chi-square test was used to compare proportions of positives. Statistical significance was set at $p < 0.05$, two-tailed.

2.6. Influenza Hemagglutinin Multiple Sequence Alignments

Multiple sequence alignments were performed using the Basic Local Alignment Search Tool (BLAST) server [21]. HA sequences of A/Brisbane/59/2007 (GenBank accession: CY030234) and A/Michigan/45/2015 (Genbank accession: MK622940) were compared against the HA sequence of A/California/07/2009 (Genbank accession: GQ280797). The similarity between sequences is expressed as a percentage.

3. Results

The results by HI and SRH assays against human influenza H1N1 vaccine strains A/Brisbane/59/2007 (referred to as A/Brisbane), A/California/07/2009 (referred to as A/California), and A/Michigan/45/2015 (referred to as A/Michigan) are shown in Figures 1 and 2.

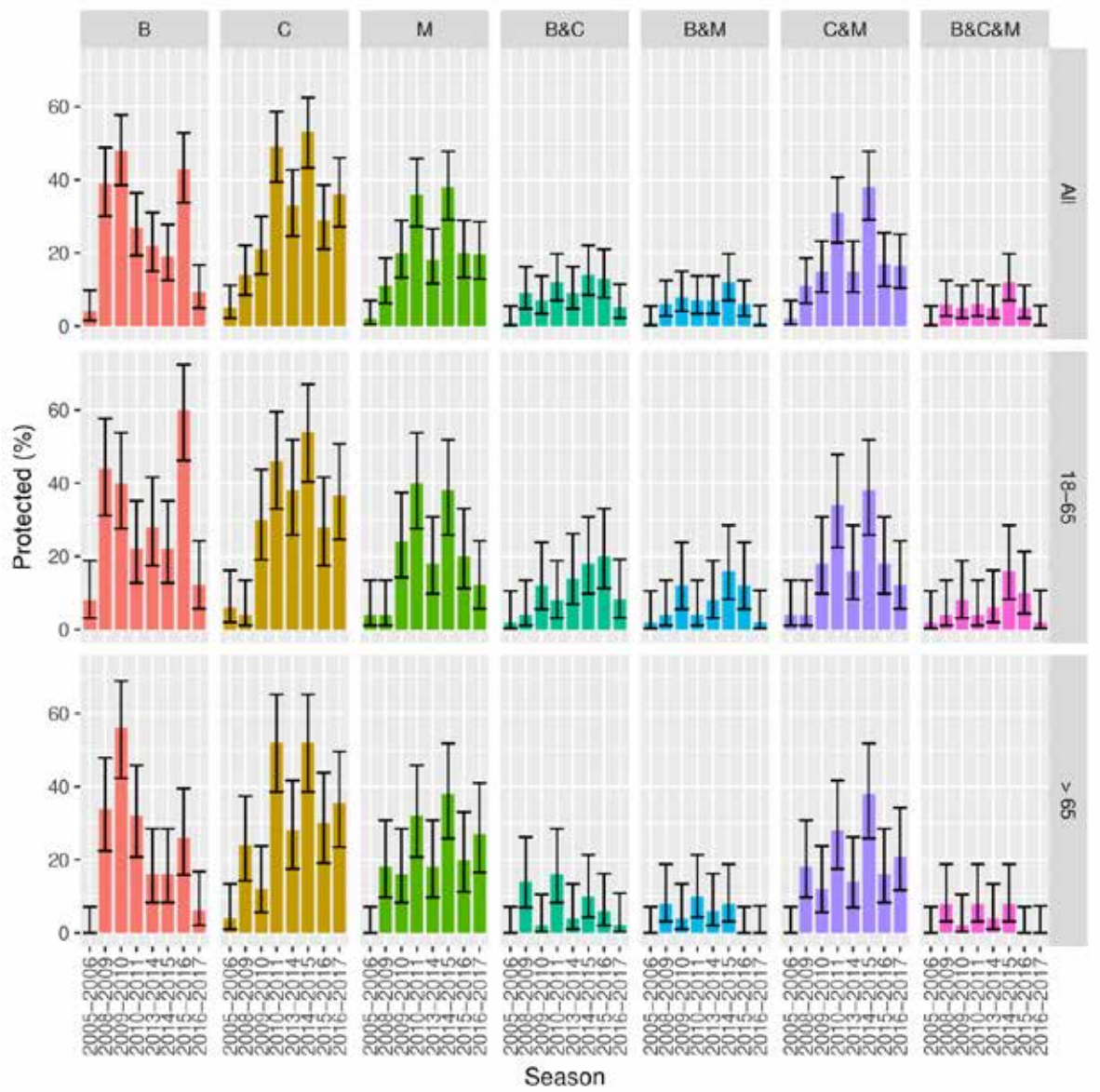

Figure 1. HI proportions of subjects with protective titers with 95% CI by strain (A/Brisbane, B; A/California, C; A/Michigan, M) and combinations by age group and season.

Between the 2005/2006 and 2008/2009 seasons, the A/Brisbane strain showed a significant increase in the proportion of positive subjects (39.0%, 95% CI 30.0–49.0 for HI and 79.0%, 95% CI 70.0–86.0 for SRH; $p < 0.0001$ for both assays). In the following years, the proportion of HI-positive subjects against the A/Brisbane strain decreased, with the exception of a peak observed for the 2015/2016 season (43.0%, 95% CI 34.0–53.0; $p = 0.0002$ vs. 2014/2015 for HI results). In contrast, variations in the proportion of positive subjects were lower in SRH.

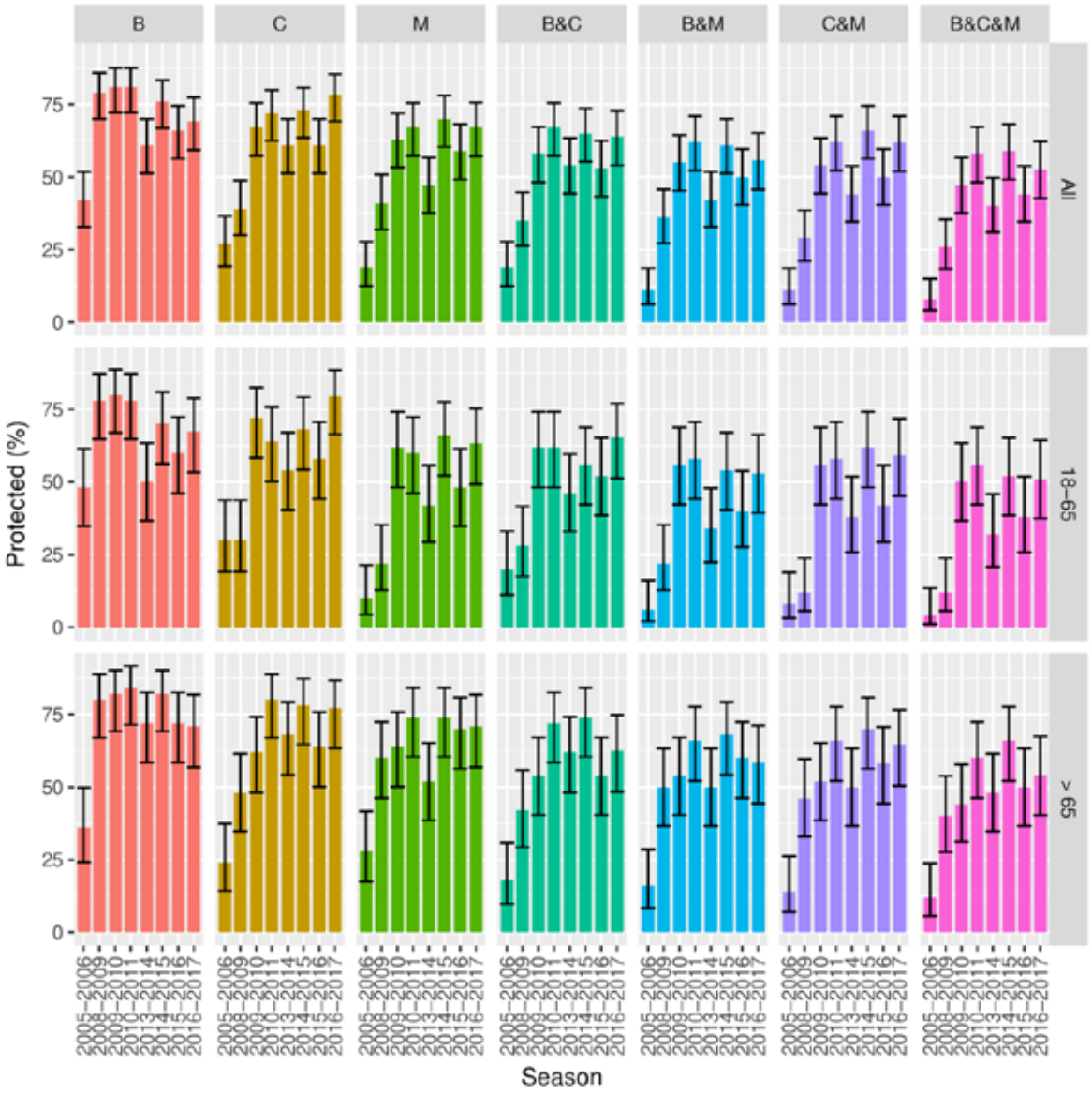

Figure 2. SRH proportions of subjects with protective titers with 95% CI by strain (A/Brisbane, B; A/California, C; A/Michigan, M) and combinations by age group and season.

The A/California strain showed a trend characterized by two different peaks: the first in 2010/2011 (49.0%, 95% CI 39.0–59.0; $p < 0.0001$ vs. 2009/2010 for HI results) and the second one in 2014/2015 (53.0%, 95% CI 43.0–62.0; $p = 0.004$ vs. 2013/2014 for HI results). As observed for the A/California strain, the A/Michigan strain also showed a peak in the 2010/2011 (36.0%, 95% CI 27.0–46.0; $p = 0.012$ vs. 2009/2010 for HI results) and 2014/2015 (38.0%, 95% CI 29.0–48.0; $p = 0.002$ vs. 2013/2014 for HI results) seasons. Considering the SRH results, both strains showed a significant increase in the 2009/2010 season (67.0%, 95% CI 57.0–75.0 for A/California and 63.0%, 95% CI 53.0–72.0 for A/Michigan; $p < 0.0001$ and $p = 0.002$ vs. 2008/2009, respectively), whereas for 2014/2015, only an increase in A/Michigan was observed (70.0%, 95% CI 60.0–78.0; $p = 0.002$ vs. 2013/2014). In general, the proportions of positive subjects for A/Michigan were lower than for A/California in both assays.

Considering the proportions of cross-protection (Figure 3A), in the 2008/2009 season HI positives were mostly positive only for A/Brisbane (30.0%, 95% CI 21.9–39.6). In the 2009/2010 season, the higher value was still represented by A/Brisbane only (38.0%, 95% CI 29.1–47.8), with a higher proportion of positives in the >65-year-old age group (52.0%, 95% CI 38.5–65.2 vs. 24%, 95% CI 14.2–37.5 in the 18–65-year-old age group, $p = 0.004$). In the same season, the second highest proportion was

observed for A/California and A/Michigan (10%, 95% CI 5.3–17.6 of cross-protection), and from the 2010/2011 season onwards, the highest proportions were for A/California and A/Michigan together or for A/California only, with the exception of the 2015/2016 season.

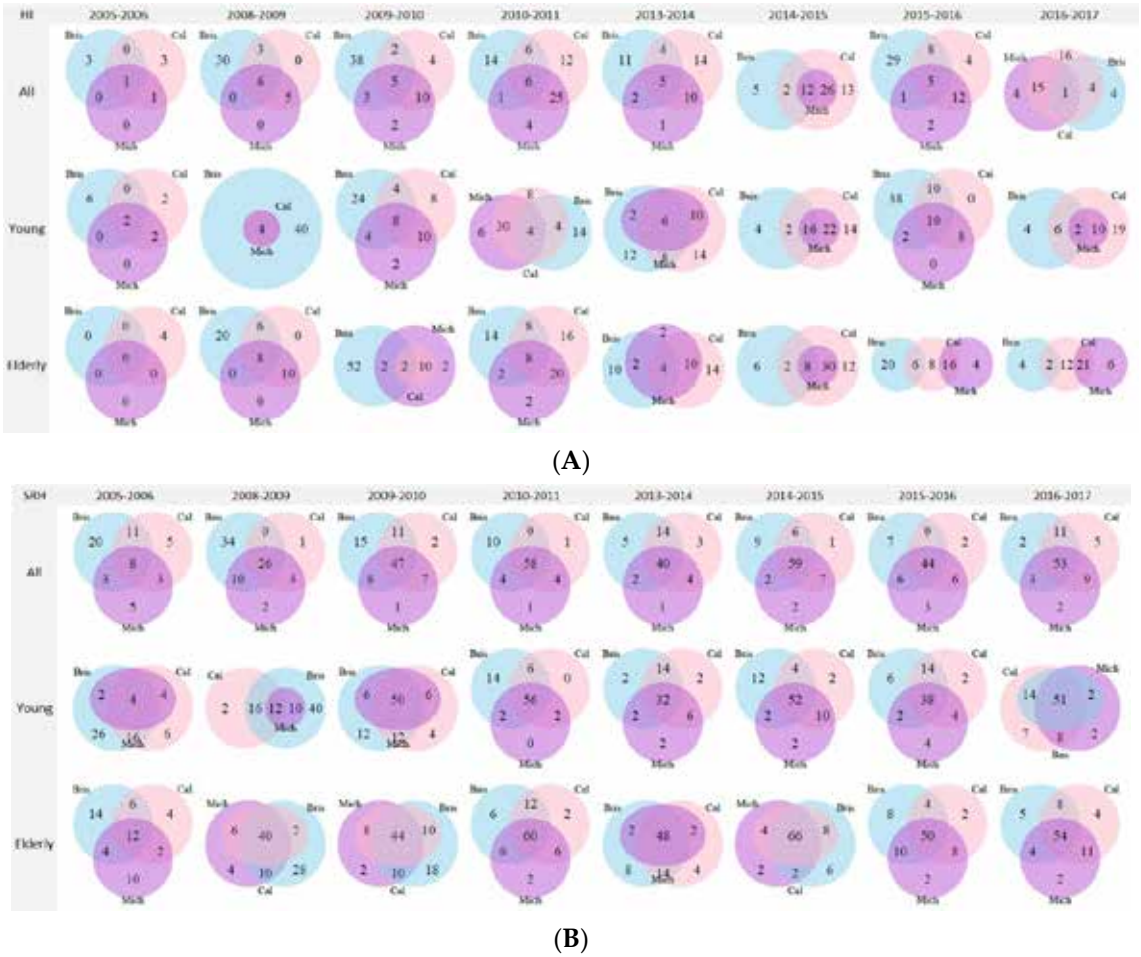

Figure 3. (**A**) Venn diagrams for HI proportions of subjects with protective titers (A/Brisbane, blue circle; A/California, pink circle; A/Michigan, purple circle) and combinations, by age group and season. (**B**) Venn diagrams for SRH proportions of subjects with protective titers (A/Brisbane, blue circle; A/California, pink circle; A/Michigan, purple circle) and combinations, by age group and season.

Considering the SRH results (Figure 3B) in the 2005/2006 season, 42.0% (95% CI 33.0–52.0) of the total population already showed protective titers against A/Brisbane, 27.0% (95% CI 19.0–36.0) for A/California, and 19.0% (95% CI 13.0–28.0) for A/Michigan. The highest proportion was for positives to A/Brisbane only (20.0%, 95% CI 13.3–29.0), followed by proportion of positives for A/California and A/Brisbane (11.0%, 95% CI 6.1–18.8). Considering the >65-year-old age group, the second highest proportion was for all three strains together (12.0%, 95% CI 5.2–24.2). In the 2008/2009 season, 34.0% (95% CI 25.4–43.7) of the subjects showed positivity to A/Brisbane only. However, when considering age groups, 40% (95% CI 27.6–53.8) of >65-year-olds showed protective titers for all of the three strains. This was also reflected in the results for the total population, where 26.0% (95% CI 18.4–35.4) of subjects had protective titers against all of three strains. Starting from the 2009/2010 season onward, in both age groups, the highest proportion of subjects had protective titers against all three strains.

Overall, proportions of subjects with protective titers were significantly higher for the SRH assay across strains, seasons, and age groups than the HI assay ($p < 0.0001$) (Table 2). The proportion of SRH positives was higher in the >65-year-olds age group in comparison to the 18–65-year-old group.

Table 2. Proportions of positives (%) by strain, age group, and assay.

Age Group	Assay	A/Brisbane	A/California	A/Michigan
18–65	HI	29.50	30.00	20.25
>65	HI	23.25	29.75	20.75
18–65	SRH	66.17	58.95	46.91
>65	SRH	72.17	63.19	61.43

A comparison between the proportion of HI and SRH positives was performed between pre- and post-outbreak seasons for each strain, with reference to the WHO vaccine strain recommendation (Table 1). Seasons 2005/2006 (pre-outbreak) and 2008/2009, and the following seasons (post-outbreak) were compared for the A/Brisbane strain, and seasons 2005/2006 and 2008/2009 (pre-outbreak) were compared to 2009/2010 and the following seasons (post-outbreak) for the A/California strain. In contrast, we found no clear definition of pre- and post-outbreak seasons for the A/Michigan strain when comparing the years before the WHO recommendation. Therefore, based on the serological results, the most reasonable season for the appearance was set as the 2014/2015 season.

HI and SRH results for A/Brisbane and A/California strains showed a clear distinction between the number of subjects with positive titers between pre- and post-outbreak seasons ($p < 0.0001$ for both assays). For the A/Michigan strain, differences between pre- and post-outbreak seasons were more evident for SRH ($p < 0.0001$) than for HI ($p = 0.01$) (Figure 4).

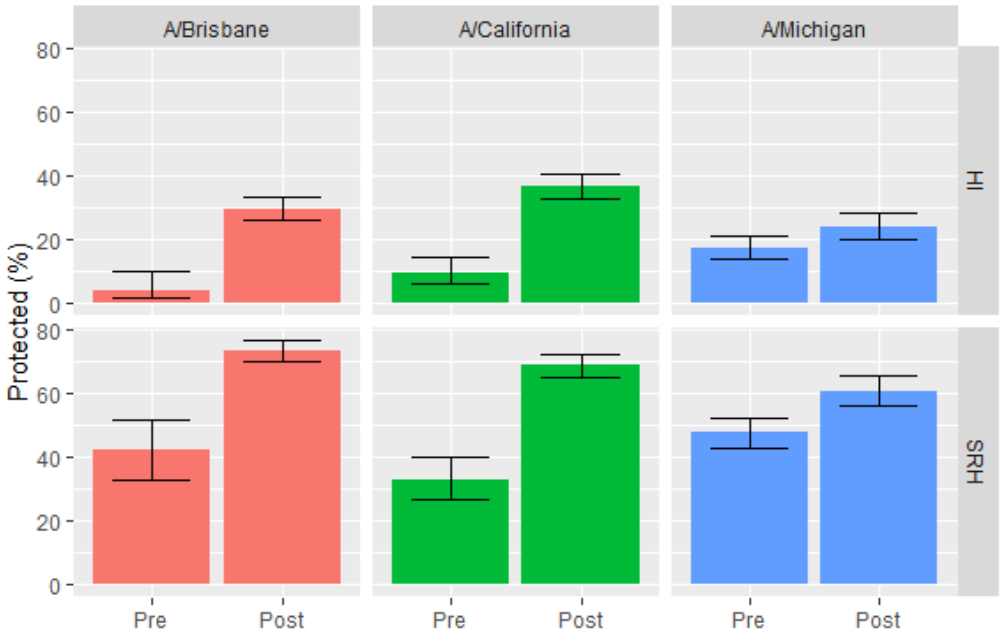

Figure 4. Pre- and post-outbreak seasons by strain. HI and SRH results.

To provide an explanation for the observed cross-protective antibody responses, the amino acid sequences of HA were compared between the strains. The HA amino acid sequence of A/California was aligned with the HA amino acid sequences from the A/Brisbane and A/Michigan strains. The A/California strain exhibited the highest sequence identity to the A/Michigan strain (97.1% homology). In particular, the HA1 domain of A/California shared 96.0% homology with the A/Michigan strain's HA1 domain, and 98.6% for the HA2 domain. In contrast, HA sequence identity to A/Brisbane was lower (79.8%), with 71.6% homology for HA1 and 91.8% for HA2.

4. Discussion

This study provided a serologically-based evaluation and interpretation of the levels of cross-reactive (and cross-protective) antibodies against H1N1 viruses circulating in the human population since 2008.

In the 2005/2006 season, low HI proportions of subjects with protective titers were observed in both age groups, as none of the H1N1 strains included in this study were circulating. The SRH results showed some protection, most probably correlating with pre-existing immunity, which was higher for A/Brisbane (42.0%) and lower for A/California and A/Michigan (27.0% and 19.0%, respectively). However, elderly adults showed higher cross-protective titers against all the three strains (12.0%) than younger adults. In the 2008/2009 season (considered pre-pandemic), the HI results clearly showed A/Brisbane as the predominant strain, especially in elderly adults (34.0%). With respect to SRH, 34.0% of the total population had protective titers against A/Brisbane; however, 40.0% of elderly adults showed cross-protective titers against all three strains, probably because of the presence of pre-existing cross-reactive antibodies against former H1N1 strains that may have contributed to protection in this age group [3]. In the 2009/2010 season, the higher proportion of protective antibodies was still A/Brisbane-specific; however, cross-protective antibodies between A/California and A/Michigan were already detectable, most likely induced by the spread of the 2009 pandemic strain. Starting from the 2009/2010 season onward, a higher proportion of SRH positive subjects showed cross-protection for all three strains. In the 2010/2011 (pandemic/post-pandemic) season and onward, higher proportions of protective antibodies were found against A/California and A/Michigan together, or A/California only, and in seasons when A/Michigan was possibly circulating.

Despite some antigenic differences, the A/California and A/Michigan strains share common epitopes. Although the WHO recommended A/Michigan as the H1N1 vaccine component in the 2017/2018 season, replacing A/California, the population already showed protection against the A/Michigan strain in the first seasons of A/California circulation. It could be concluded that there has been a co-evolution of both strains over the seasons, associated with a cross-reactivity between A/California and A/Michigan that does not allow a discrimination of the exact season when A/Michigan strain became predominant. The data described here indicate that antibodies raised against A/California are cross-protective against A/Michigan. As previously observed [22,23], the antigenic drift of HA of the 2009 pandemic H1N1 strain was not observed with ferret antisera, although more recent 2009 pandemic H1N1-like strains, such as A/Michigan, are antigenically different to the vaccine strain (A/California).

In elderly adults, protective antibody titers against A/Brisbane strain were associated with cross-protective antibodies against A/Michigan and A/California strains as well. One possible explanation is that, despite the HA from the pandemic H1N1 virus not being antigenically similar to any previous human seasonal influenza virus [3], the adaptation to human hosts led to common conserved epitopes, which can be recognized by the broader antibody repertoire of elderly adults and, as such, result in higher titers in this age group. This is also supported by the observation that although the elderly may show lower titers than younger adults against homologous strains, they showed higher cross-reactions (and cross-protective titers) against heterologous strains (drifted strains). These findings were supported by other studies [9,11], which have shown that immunological priming by previous exposure to influenza strains participates in the immune response to an antigenically-related strain and increases with age.

The analysis performed in this study supports the use of the two different assays, HI and SRH, in parallel, as it allows investigations into the antigenic nature of three different virus types in two distinct populations, younger and elderly adults, in an unusual scenario; (i) a seasonal influenza H1N1 strain of human origin as a representative of classical drifted A/H1N1 strains, A/Brisbane; (ii) an influenza H1N1 virus of animal origin (swine) that caused a pandemic in humans in 2009/2010 and then became a classical seasonal human influenza virus replacing the former seasonal human H1N1 strain, A/California; and (iii) a drift variant of the original pandemic strain due to antigenic

changes resulting from frequent circulation in the human population over several seasons, A/Michigan. This study showed that the HI assay is an optimal assay for determination and detection of new seasonal drift strains, as shown in the pre/post-outbreak A/Brisbane and pre/post-outbreak A/California seasons. Based on its specificity, the HI assay is able to discriminate which new strain is circulating following vaccine recommendation. In accordance with the HI results of this study in both age groups, it is possible to recognize peaks of prevalence through the seasons that, in the majority of cases, are in accordance with virologic surveillance data. SRH immunity seems to accumulate over the seasons, with minor variations in the proportion of positive subjects. Antibodies against viral epitopes, recognized by SRH, are more stable between seasons and strains, as the SRH assay is able to detect antibodies directed against potentially more conserved epitopes between different strains, such as antibodies against HA2 [15], allowing the detection of a broader range of functional antibodies that contribute to previous immunity. This previous immunity was observed to be more pronounced in the elderly, as already highlighted by previous studies [3,7–11]. The SRH assay, in addition, is an excellent tool for determining the presence of pre-existing immunity against drifted and actual circulating seasonal strains. This would allow a potential prediction of booster ability against newly emerging drifted strains.

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Influenza Anti-Stalk Antibodies: Development of a New Method for the Evaluation of the Immune Responses to Universal Vaccine

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Abstract: Growing interest in universal influenza vaccines and novel administration routes has led to the development of alternative serological assays that are able to detect antibodies against conserved epitopes. We present a competitive ELISA method that is able to accurately determine the ratio of serum immunoglobulin G directed against the different domains of the hemagglutinin, the head and the stalk. Human serum samples were treated with two variants of the hemagglutinin protein from the A/California/7/2009 influenza virus. The signals detected were assigned to different groups of antibodies and presented as a ratio between head and stalk domains. A subset of selected sera was also tested by hemagglutination inhibition, single radial hemolysis, microneutralization, and enzyme-linked lectin assays. Pre-vaccination samples from adults showed a quite high presence of anti-stalk antibodies, and the results were substantially in line with those of the classical serological assays. By contrast, pre-vaccination samples from children did not present anti-stalk antibodies, and the majority of the anti-hemagglutinin antibodies that were detected after vaccination were directed against the head domain. The presented approach, when supported by further assays, can be used to assess the presence of specific anti-stalk antibodies and the potential boost of broadly protective antibodies, especially in the case of novel universal influenza vaccine approaches.

Keywords: hemagglutinin; stalk domain; HA2-antibody; competitive ELISA; universal influenza vaccine

1. Introduction

Influenza continues to have a significant impact on public health and is still responsible for high morbidity and mortality in humans, with annual attack rates estimated to be up to 10% in adults and 30% in children [1]. Vaccination is still the most effective method of preventing the morbidity and mortality caused by influenza infection, especially in groups at high risk of dangerous complications, such as young children and the elderly [2], although the effectiveness of influenza vaccination is strictly dependent on the age-group and vaccine formulation [3,4]. Influenza A and B viruses, which are responsible for annual epidemics in humans, undergo antigenic changes within the antibody-binding sites of the hemagglutinin (HA) and neuraminidase (NA) antigens; these changes are able to render the new strains different enough to at least partially avoid the immunity

induced by previous infection or vaccination (antigenic drift) [5,6]. Consequently, the composition of vaccines needs to be updated every year in response to changes in HA antigens. Current inactivated intramuscular/intradermal vaccines (IIVs) or live attenuated influenza vaccines (LAIVs) are made with a carefully standardized amount of HA from three (trivalent influenza vaccine—TIV) or four (quadrivalent influenza vaccine—QIV) seasonal strains on the basis of recommendations by the World Health Organization (WHO) [7,8]. Despite the efforts of the WHO Collaborating Centers and the new mathematical modelling approach [6,9] to monitor antigenic drift, an intrinsic uncertainty concerning the match between the circulating viruses and the vaccine strains remains [10].

Another important consideration is the fact that the currently available influenza vaccines are not able to protect against emerging pandemic-like influenza viruses [11]. Moreover, with today's manufacturing technologies, it would take at least six-to-eight months to prepare a new vaccine; in the event of urgent necessity, this may be too long, as demonstrated by the 2009 H1N1 pandemic [12,13].

The development of a universal influenza vaccine would avoid potential mismatches of recommended vaccine strains and the need for the annual re-formulation and re-administration of vaccines; it would also enable timely intervention in the event of a pandemic, and it might result in the eradication of influenza B virus in humans. Several candidate target antigens could be considered for use in universal influenza vaccines, such as the M2 ion channel [14], NA [15], and conserved regions of the head domain (HA1) [16] and stalk domain (HA2) of HA [17]. The stalk domain is the most conserved region of HA in the influenza A and B viruses. Its main function is to mediate the fusion of the viral and endosomal membranes once the virus has been internalized by endosomes in order to permit the release of the viral genome into the cytosol [18]. In order to carry out this function, the stalk domain has to undergo considerable structural rearrangements; this is why all possible mutations that could potentially interfere with this process are not permitted [17].

Classically, antibody-mediated immune responses after influenza vaccination or natural infection are assessed by standard serological assays such as hemagglutination inhibition (HI), single radial hemolysis (SRH), and micro-neutralization (MN) [19]. These methods are recommended by regulatory authorities and are considered the gold standard in detecting the immune response in serum samples. The HI assay detects antibodies that bind to the viral HA and prevent the agglutination of red blood cells (RBCs) by blocking the receptor binding site. The MN assay identifies functional neutralizing antibodies, including those that recognize epitopes in the stalk region of HA, which are conserved among different subtypes of influenza A viruses. The SRH assay may recognize not only antibodies against the surface glycoproteins but also those against the internal antigens [20]. However, these assays are generally insufficient to detect the immune response after immunization with LAIVs or conserved epitope-based vaccines. Moreover, HI titers are not always able to predict the right degree of protection from a disease, especially in children [21], the elderly [22] and obese subjects [23].

The growing interest in developing a universal influenza vaccine has led to the need for alternative serological assays that are able to detect different classes of antibodies, such as anti-stalk, anti-NA, and secretory immunoglobulin A (s-IgA) ones [24]. Stalk-specific antibodies can be detected mainly by enzyme-linked immunosorbent assays (ELISAs) by using purified chimeric (cHA) proteins, such as cH6/1 (which contains an H6 head domain from A/mallard/Sweden/81/02 combined with an H1 stalk domain of A/California/04/09) [25].

ELISAs, including the competitive assay described in the present study, are not able to predict whether the antibodies detected are functional. In order to support the results of ELISA, other assays can be adapted on the basis of some functions that anti-stalk antibodies can exert through various mechanisms, such us neutralization, Fc receptor activation, and NA inhibitory activity [26].

In this paper, we present a potential method of indirectly detecting specific anti-stalk serum immunoglobulin G (IgG) antibodies against conserved epitopes among group 1 and group 2 influenza A viruses by measuring the difference between the HA head and total HA response; this method, based on a re-adaptation of a competitive ELISA, allows for the discrimination and the quantification of antibodies that are directed against the head and stalk subunits. The construction of a stable headless

HA would be an important step both for universal vaccine studies and serological assay use. There have been several studies [27–29] that were animated by the quest to find a stable form of an HA stalk without the head domain, but the correct stabilization and folding of the constructs remain to be evaluated in more detail. In this study, we evaluate the performance of the assay by measuring anti-head and anti-stalk responses in a small panel of human serum samples (adults and children) taken before and after vaccination in the 2009/2010 season.

2. Materials and Methods

2.1. Virus Antigen

The virus antigen and infectious influenza virus was the seasonal influenza strain A/California/7/2009 H1N1 (15/252), grown in eggs and obtained from NIBSC, UK.

2.2. Pseudotype Production

Lentiviral pseudo-virus particles (PVs) were produced by co-transfecting Human Embryonic Kidney (HEK) 293T/17 (ATCC®CRL-11268™) cells with phCMV1-H11 (H11 from A/ruddy turnstone/New Jersey/650653/2002 (H11N9)) and pI.18-N1Cal/09, as previously described. The H11 plasmid was added to make the NA more stable and to increase PV release and production. Briefly, 1 μg of HA, 1 μg of NA and 1.5 μg pNLLuc4.3 plasmids were transfected into HEK293T/17 cell lines by means of Endofectin™ Lenti (3μL/μg). The medium was changed 24 hours after transfection, and PVs were harvested after 48 hours. The titration of the NA activity of each PV was performed in an enzyme-linked lectin assay (ELLA), as in the protocol reported by Biuso et al. [30].

2.3. Serum Samples

Human serum samples (n = 48; obtained before and after vaccination) were kindly provided by the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena, where they had been stored in compliance with Italian ethics law. The following information was available for each serum sample: adult (18+ years) or child (3–9 years) age-group, year of sampling (2009–2010), and pre- and post-vaccination withdrawal.

2.4. Hemagglutination Inhibition Assay

Serum samples were pre-treated with a receptor-destroying enzyme (RDE—Denka Seiken) for 18 hours at 37 °C in a water bath and then heat-inactivated for 1 hour at 56 °C in a water bath. At the end of incubation, all serum samples were treated with a 10% turkey RBCs (TRBCs) solution in order to remove non-specific inhibitors, and they were run in the HI assay by using the A/California/7/2009 H1N1pdm09 influenza strain, as described elsewhere [31]. HI titers below 10 were assigned a titer of 5 and considered negative.

2.5. Single Radial Hemolysis Assay

Serum samples were heat-inactivated at 56 °C for 30 minutes in a water bath before testing. Then, 6 μL of each serum sample was tested in SRH plates that were prepared in accordance with the protocol described by Trombetta and colleagues [32] in which the virus antigen was diluted at 2000 hemagglutinin units per milliliter in a TRBC suspension and guinea pig complement. The diameters of hemolysis were read in millimeters by a dedicated calibrating viewer.

2.6. Micro-Neutralization Assay

The MN assay was performed as described previously [33]. Briefly, heat-inactivated serum samples were mixed and incubated for 1 hour at 37 °C and 5% CO_2 in a humidified atmosphere with a standardized amount of live A/California/7/2009 H1N1 influenza virus (100 tissue culture infective dose 50% (TCID50)). After the incubation period, the serum–virus mixtures were transferred to a plate

that contained 90% confluent pre-seeded Madin–Darby canine kidney (MDCK) (ATCC®CCL-34™) cells that were monolayered in an UltraMDCK serum-free medium (Lonza, Milano, Italy) with 7 μg/mL of acetylated trypsin (Sigma, St. Louis, MO, USA). The plates were then incubated for 5 days at 37 °C and 5% CO_2 in humidified atmosphere before being inspected by an inverted optical microscope for the presence/absence of a cytopathic effect (CPE).

2.7. Enzyme-Linked Lectin Assay

Anti-NA antibodies were also determined by the ELLA assay in accordance with the protocol described by Couzens and colleagues [34]. Briefly, inactivated and 2-fold diluted serum samples were mixed with a standardized amount of influenza pseudotypes bearing N1 from A/California/7/2009, and incubated for 16–18 hours in a fetuin- (Sigma, St. Louis, MO, USA) coated plate. After the incubation period, the plates were washed, and peanut agglutinin (PNA) that was conjugated to horse-radish peroxidase (HRP) (Sigma, St. Louis, MO, USA) was added to all wells. After 2 hours of incubation, the plates were washed, and an o-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, MO, USA) substrate was added. The reaction was stopped, and the absorbance was read at 490 nm.

2.8. Competitive ELISA for Anti-HA2 Antibody Detection

The competitive ELISA procedure described here (Figure 1) utilized the ELISA Starter Accessory Kit (Bethyl Laboratories, Montgomery, TX, USA). ELISA plates were coated with purified recombinant HA (aa 18–529) (eEnzyme, Gaithersburg, MD, USA); serum samples were incubated with purified recombinant HA (aa 18–529) and head (aa 18–345) proteins from the A/California/7/2009 H1N1 influenza virus (eEnzyme, Gaithersburg, MD, USA). A solution of 5% non-fat dried milk (NFDM; Euroclonelone, Pero, Italy) in 0.05% Tris buffered saline-Tween 20 (TBS-T) (Thermo Scientific, Rodano, Italy) was used for plate blocking. ELISA 96-well plates were coated with the HA protein at a concentration of 1 μg/mL and incubated overnight at 4 °C. For each serum sample tested, three incubation conditions were prepared: 1) the HA recombinant protein in serial dilutions; 2) the head recombinant protein in serial dilutions; and 3) the TBS-T buffer without a protein, which was used for treatment control. Series of two-fold dilutions of HA and head proteins in TBS-T were prepared in rows of dedicated 96-well dilution plates. The starting concentration of the protein was 75 μg/mL in the first well, each well containing a volume of 20 μL of the solution. For each sample tested, one control row of wells containing 20 μL of the buffer (without HA or head proteins) was prepared. Serum samples that were designated for treatment were pre-diluted in TBS-T (1:250) and subsequently added to the prepared incubation rows in a 1:1 ratio; the protein concentration in each incubation well was halved in order to obtain a final serum dilution of 1:500. Reaction plates were incubated for 2 hours at 37 °C. Next, 60 μL of the TBS-T buffer were added to each well containing 40 μL of the serum solution. At the end of this step, each well contained 100 μL of serum that were diluted to 1:1250. Coated plates were washed three times with 300 μL/well of an ELISA washing solution. Plates were blocked and incubated at 37 °C for 2 hours. Blocked plates were washed 3 times with 300 μL/well of washing solution. Subsequently, 95 μL of prepared serum samples from incubation plates were transferred into the corresponding wells of the ELISA plate by means of a multichannel pipette. Experimental plates were covered and incubated at 37 °C for 1 hour. Next, the plates were washed as previously stated, and 100 μL/well of goat, anti-human IgG-Fc HRP-conjugated antibody (Bethyl Laboratories, Montgomery, TX, USA) was added. Plates were incubated at 37 °C for 1 hour. Following incubation, the plates were washed, and 100 μL/well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Bethyl Laboratories, Montgomery, TX, USA) was added and incubated in the dark at room temperature for 30 minutes. The reaction was stopped by adding 100 μL of an ELISA stop solution (Bethyl Laboratories, Montgomery, TX, USA), and then it was read within 20 minutes at 450 nm. Optical density (OD) values were used to draw a graph that confirmed the saturation of the samples with the protein (values reaching the lower plateau of the plot should have been seen in the samples that were treated with the highest concentration of the

recombinant protein). Next, a blank OD was subtracted from all raw data results. The results from each serum sample in the three conditions (HA, head and no protein) were selected for stalk OD calculation. For these calculations, only 4 wells (at the lower plateau OD level) were used (e.g., the first four wells with the highest head (HA1) and HA protein concentrations). Results were calculated as follows:

$$\mathbf{OD.HA2} = \overline{\mathbf{OD}}\mathbf{HA1} - \overline{\mathbf{OD}}\mathbf{HA}$$

$$\mathbf{OD.HA} = \overline{\mathbf{OD}}\mathbf{NT} - \overline{\mathbf{OD}}\mathbf{HA}$$

$$\mathbf{OD.HA1} = \left(\overline{\mathbf{OD}}\mathbf{NT} - \overline{\mathbf{OD}}\mathbf{HA}\right) - \left(\overline{\mathbf{OD}}\mathbf{HA1} - \overline{\mathbf{OD}}\mathbf{HA}\right)$$

Where $\overline{\text{OD}}$HA1 is the average OD of samples incubated with the head (HA1) protein, $\overline{\text{OD}}$HA is the average OD of samples incubated with the HA protein, and $\overline{\text{OD}}$NT is the average OD of samples incubated with the buffer (non-treated samples).

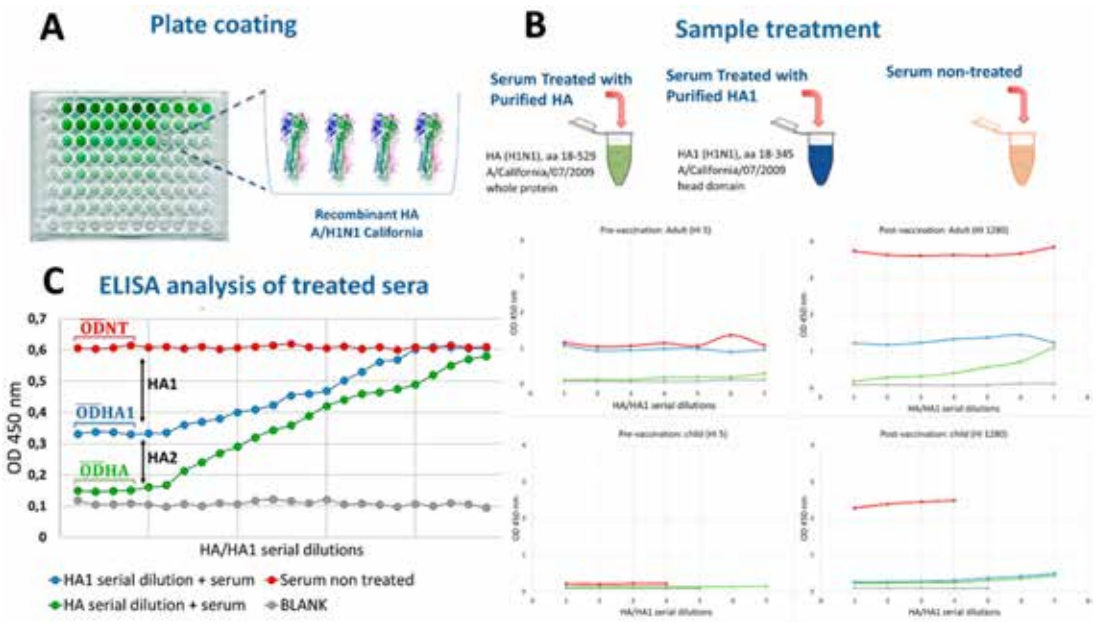

Figure 1. Schematic overview of the competitive ELISA method. (**A**) ELISA plates were coated with a purified hemagglutinin (HA) recombinant protein from A/California/7/2009 (H1N1) influenza strain. (**B**) A 1:250 pre-diluted serum sample is treated and incubated with different HA and HA1 concentrations. (**C**) The resulting OD difference between the highest head domain (HA1)-treated and the HA-treated sample can be attributed to the stalk domain (HA2) response. Two examples of treatment are reported, with appreciable pre-vaccination differences in the HA2 response between adults and children. $\overline{\text{OD}}$HA1 is the average optical density (OD) of the samples that were incubated with the head protein (HA1); $\overline{\text{OD}}$HA is the average OD of the samples that were incubated with the HA protein; and $\overline{\text{OD}}$NT is the average OD of the samples that were incubated with the buffer (non-treated samples).

2.9. Statistical Analysis

Data were analyzed by GraphPad Prism. The ELLA, SRH and MN results were normalized by applying the Z-Score. Significant differences between pre- and post-vaccination OD signals (a value of 4 at the lower plateau level) for the head and stalk were evaluated with a paired T-test. The homogeneity of variances was previously verified through an F-test. A significance level of 5% was considered for all the statistical tests.

3. Results

3.1. Serum Samples Were Selected Based on HI Titers

Human serum samples that were obtained before and after vaccination were used in this study. As a proof-of-concept to evaluate the performance of ELISA in distinguishing between head- and stalk-specific differences, a total of 16 pairs of serum samples (pre-/post-vaccination) from adult subjects and eight pairs of serum samples (pre-/post-vaccination) from children were selected on the basis of their HI titers. In the first run of experiments, we selected eight adults and four children. Among the adults, we selected: one subject in whom the HI assay gave negative results both pre- and post-vaccination (5/5); three subjects with negative pre-vaccination HI titers and seroprotective post-vaccination HI titers (5/40); three subjects with very high boost (5/1280) of HI titers after vaccination; and one subject with a pre-existing HI titer of 160 which only marginally increased to 320 after vaccination. These selected samples were titrated by serological assays that are generally used in order to evaluate the immunogenicity of an influenza vaccine (MN, SRH and ELLA), along with the competitive head/stalk-specific ELISA described here (Table 1 and Figure 2A). The above-described serological analysis was repeated on the four samples from children, who had a pre-vaccination HI titer of 5 and post-vaccination HI titers of 80, 226.3, 320 and 380 (Table 2 and Figure 3A). We decided to investigate the immune response and the accuracy of the new ELISA method in a small number of children, too, because we expected to find significant differences in the stalk response between the two age-groups (adults and children) as a result of the previous exposure and/or vaccination of the adults. In the second run of experiments, to broaden our view of the variation in anti-head/stalk responses in individual subjects, we evaluated the performance of the head/stalk-specific ELISA on another eight pairs of samples from adults and four pairs of samples from children with different pre- and post-vaccination HI titers (Figures 2B and 3B).

Table 1. Samples from adult subjects tested by the HI, ELISA, ELLA, SRH and MN assays.

Subject	Dose	HI Titer	Competitive ELISA			ELLA Titer	SRH Area [mm2]	MN Titer
			OD Stalk (HA2)	OD Head (HA1)	OD HA			
1	Pre	5	0.187	0.133	0.320	10	10.2	40
	Post	5	0.182	0.295	0.477	15	17.3	40
2	Pre	5	0.133	−0.018	0.116	5	11.3	20
	Post	40	0.220	0.069	* 0.289	10	19.6	30
3	Pre	5	0.281	0.022	0.303	10	2.256	20
	Post	40	0.391	* 0.255	* 0.647	80	2.256	20
4	Pre	5	0.238	0.032	0.269	80	2.256	20
	Post	40	0.211	0.159	0.370	640	60.8	20
5	Pre	160	0.723	1.164	1.887	160	38.5	40
	Post	320	* 1.041	* 1.834	* 2.875	320	50.2	80
6	Pre	5	0.341	0.059	0.400	5	2.256	40
	Post	1280	0.485	* 1.464	* 1.949	40	63.6	320
7	Pre	5	0.452	0.039	0.491	20	2.256	20
	Post	1280	* 1.499	0.113	*1.612	160	63.6	40
8	Pre	5	0.816	0.131	0.947	40	2.256	40
	Post	1280	* 0.987	* 2.371	* 3.359	1280	107.5	640

In the ELLA, HI and MN assays, titers below 10 were assigned a value of 5 and considered negative. In an SRH assay, samples which did not show hemolysis were assigned an area value of 2.256 mm^2. Statistically significant increases in OD stalk or OD head post-vaccination are marked with an asterisk.

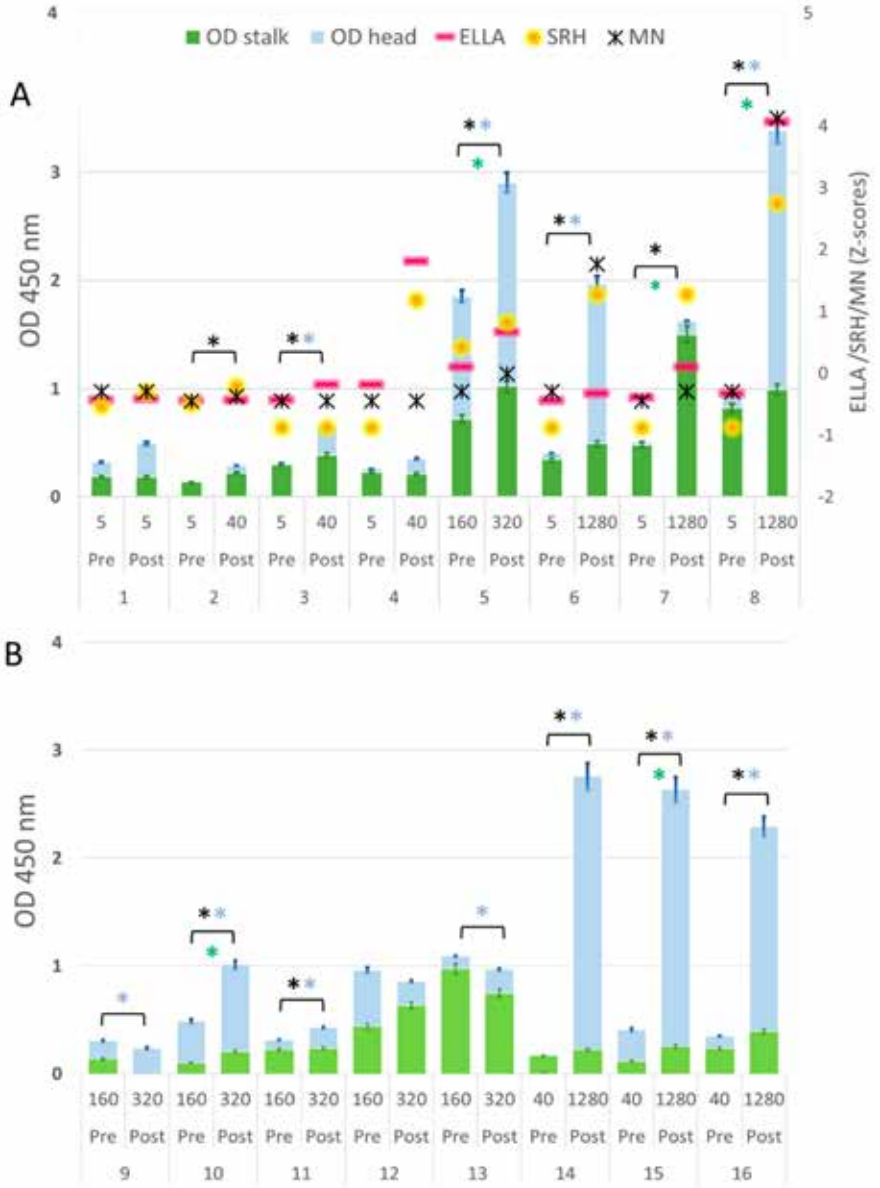

Figure 2. (**A**) Serum samples from adult subjects tested by ELISA, hemagglutination inhibition (HI), single radial hemolysis (SRH), micro-neutralization (MN) and enzyme-linked lectin (ELLA) assays; (**B**) Serum samples from adults tested by ELISA and HI assays. The HI titer of each sample is indicated below the x-axis. Blue bars = head signal, and green bars = stalk signal. Asterisks indicate statistical significance; a black asterisk indicates a significant increase in the HA signal; a blue asterisk indicates a significant increase in the head signal; a green asterisk indicates a significant increase in the stalk signal. Error standard bars are reported both for the head and stalk signals.

Table 2. Samples from children tested by the HI, ELISA, ELLA, SRH and MN assays.

Subject	Dose	HI Titer	Competitive ELISA			ELLA Titer	SRH Area [mm2]	MN Titer
			OD Stalk (HA2)	OD Head (HA1)	OD HA			
1	Pre	5	0.006	0.007	0.014	5	2.256	40
	Post	80	0.039	* 0.179	* 0.219	120	21.2	80
2	Pre	5	−0.016	0.023	0.007	5	2.256	20
	Post	226.3	* 0.040	* 0.461	* 0.501	10	38.5	80

Table 2. *Cont.*

Subject	Dose	HI Titer	Competitive ELISA			ELLA Titer	SRH Area [mm2]	MN Titer
			OD Stalk (HA2)	OD Head (HA1)	OD HA			
3	Pre	5	0.015	0.037	0.051	5	12.6	10
	Post	320	0.009	* 0.521	* 0.530	160	59.4	80
4	Pre	5	0.016	0.044	0.060	5	2.256	40
	Post	380	* 0.251	* 0.824	* 1.075	20	50.2	160

In the HI, ELLA and MN tests, titers below 10 were assigned a value of 5 and considered negative. In SRH, samples which did not show hemolysis were assigned an area value of 2.256 mm^2. Statistically significant increases in OD stalk or OD head post-vaccination are marked with asterisk.

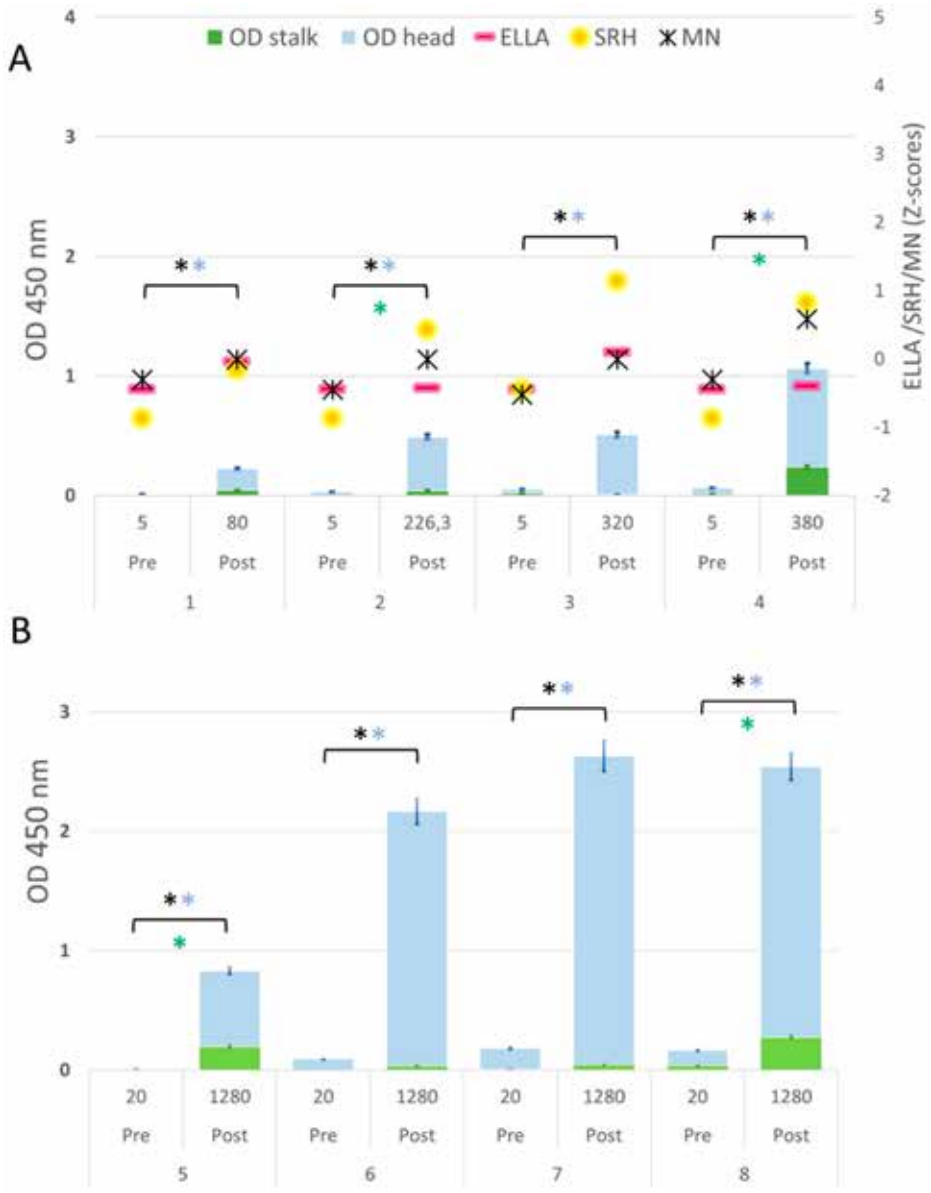

Figure 3. (**A**) Serum samples from children tested by ELISA, HI, SRH, MN and ELLA assays; (**B**) Serum samples from children tested by ELISA and HI. The HI titer of each sample is indicated below the x-axis. Blue bars = head signal, and green bars = stalk signal. Asterisks indicate statistical significance; a black asterisk indicates a significant increase in the HA signal; a blue asterisk indicates a significant increase in the head signal; a green asterisk indicates a significant increase in the stalk signal. Error standard bars are reported both for head and stalk signal.

3.2. Different Levels of Anti-HA2-specific Antibody Responses Were Found in Pre-Vaccination Samples from Adults, but Not in Children

We investigated the presence of anti-stalk antibody response in pre- and post-vaccination serum samples from adults and young children. All pre-vaccination samples from adults presented detectable stalk-specific antibodies (green bars in Figure 2A,B). The highest pre-vaccination levels assigned to the stalk antibodies were found in adult subjects 8 and 13, but they were completely independent from the measured HI titers of 5 and 160, respectively, which still correlated with the specific head response. In contrast to the results obtained in adults, no antibody responses against the stalk domain were detected in pre-vaccination pediatric serum samples when using the competitive ELISA, with the exception of subject 8 (Figure 3A,B); moreover, in both children and adults, the increased post-vaccination OD signal arose mainly from the head response (blue bars Figures 2 and 3). Heterogeneous levels of anti-head antibody signals were detected in all samples; no head signals were found in samples 2 and 14 (adults) and sample 1 (children). The magnitude of the head response detected by ELISA after vaccination generally agreed with the increase that was registered by the HI assay, apart from three cases that were observed in adult subjects 1, 7 and 14. In subject 1, a post-vaccination increase in head antibodies was seen in the ELISA, but this was not seen in the HI assay, which remained negative after vaccination. Interestingly, we identified two adult subjects with positive HI titers of 1280 after vaccination (subject 7) or of 40 before vaccination (subject 14), though they had very low, or even undetectable head responses. These quite striking observations can most likely be attributed to the high level of antibodies against conserved epitopes, a response that may be able to result in an effective steric hindrance of hemagglutination activity.

3.3. Correlation between Anti-HA1 ELISA and SRH- and MN-Antibody Responses

Given that serological assays such as MN and SRH are not able to distinguish between antibodies against the head and stalk subunits of the HA, we compared the results yielded by the ELISA with those obtained with the aforementioned methods. The SRH data appeared to be more in line with the anti-head response detected by the ELISA and HI assay than with the titers measured by the MN assay. In all adults and children assessed by the SRH assay (yellow dot in Figures 2A and 3A), we were able to detect a post-vaccination increase in the hemolysis area, apart from adult subject 3, who showed no increase in SRH but did show an HI seroconversion and an increase in the head response. By contrast, the MN assay seemed to be more specific than the SRH assay; it was possible to detect at least a two-fold increase in the neutralizing titer only in subjects that showed a greater increase in head response in the ELISA (adult subjects 5–8 and child subjects 1–4). For a more comprehensive overview on the immunological characterization of each subject, we also evaluated the anti-NA antibody response by using the ELLA test. We found that the NA response, as expected, generally did not correlate with the anti-head or the anti-stalk response. This was clearly seen in adult subjects 4 and 6. Subject 4 showed an eight-fold increase in ELLA but no increase in MN or stalk antibody responses and only quite low increases in HI and head responses. However, subject 6 did not show a post-vaccination increase in the NA antibody titer, despite high responses in the HI, MN and head ELISA tests. These results confirmed that the immunological responses against NA could not be related to or predicted by the HA responses.

4. Discussion

Along with vaccination coverage, which remains unsatisfactory [35], one of the main drawbacks of the current influenza vaccines is the need for an annual reformulation and consequent global re-administration, owing to the antigenic drift of the influenza virus. In the last two decades, growing interest in the possibility of developing a universal vaccine has given new impetus to influenza research. Several studies have focused on the extracellular domain of the M2 protein [36,37]. The ectodomain sequence has proven to be highly conserved among human and avian influenza viruses. However, antibodies that are elicited against this conserved portion are not neutralizing, but, due to the high expression of M2 on the surface of infected cells, they can promote protection through the effector

function of their Fc region [38]. NA, the second most abundant glycoprotein that is present on the surface of the influenza virus, is another important target. Previous murine studies that were conducted with virus-like particles bearing the N1 antigen showed protection against lethal infection by homologous and heterologous strains [39]. Compared with the immunodominant globular head, the stalk domain is far less variable and is able to induce broadly neutralizing antibodies. The first description of a mouse monoclonal antibody that was specific for the stalk domain (C179) dates back to 1993 [40]. This antibody showed no HI activity; however, it was capable of neutralizing group 1 viruses (H1 and H2). In recent years, promising research has been carried out with a view to developing a stalk-based universal influenza vaccine; this research has mainly been based on a novel approach involving the construction of cHA molecules. Repeated vaccination with these constructs has been highly effective in boosting the antibody response against conserved regions of the stalk domain, resulting in high anti-stalk titers and a reduction of viral titers in lungs and nasal turbinates in mice and ferrets [16]. A universal influenza vaccine that is able to stimulate stalk-specific antibodies has the potential to avoid the need for the annual vaccine reformulation of the H1, H3 and B strains; moreover, it would confer greater protection against new emerging influenza viruses, particularly those that pose a pandemic threat [41]. In this paper, we present a possible approach that allows for head- and stalk-specific antibody responses to be clearly distinguished through the specific re-adaptation of a competitive ELISA. Unlike the HI, SRH, MN assays or the ELLA, which detect functional antibodies, this adapted ELISA only detects binding antibodies. Nevertheless, it can support studies of the immunogenicity of influenza vaccines by detecting and quantifying specific immune responses against mainly continuously changing epitopes in the head domain of the HA molecule (antigenic drift) and mainly conserved epitopes in the stalk region. This approach will be particularly helpful for the study of the immune responses that are induced by next-generation influenza vaccines, such as those based on conserved epitopes from the stalk domain of the HA protein [28,42,43].

The classical serological assays listed above are not able to detect and distinguish specific antibodies directed against the stalk region. Though, since February 2017, the new European Medicines Agency (EMA) guidelines have withdrawn the concept of the traditional correlates of protection for influenza, the HI titer is still considered the gold standard, and the correlates of protection based on this are still used in many countries, such as the U.S., Japan and Australia [44,45].

Here, we present the results obtained from a small number of samples selected on the basis of their HI titers. The ELISA IgG signal that was obtained against the HA protein agrees with those obtained with HI and SRH assays, for which we observed a better correlation (HI–SRH) (R^2 = 0.70) to HI–MN and MN–SRH with R^2 values of 0.55 and 0.3, respectively. Despite the low number of samples that were analyzed in the present work, these results seem to confirm previous studies that have supported strong agreement between the HI and SRH assays [46,47] in respect to the higher correlation found by Wang et al. [48] between the SRH and MN. The MN assay generally suffers from high interlaboratory variability due to the lack of common protocols (long vs short/CPE vs. ELISA-based) and discrepancies in endpoint determination. On the other hand, although ELISAs are not officially acknowledged by EMA and other regulatory authorities, they usually provide unbiased and precise results. The responses that were obtained in the two age groups support the statement that the described ELISA-based assay is able to distinguish between immunological responses against head and stalk epitopes in adults and children in a very selective manner. Moreover, inside each group (adults and children), the assay is able to reveal subtle differences in HA-specific responses. Upon comparing the results obtained in children and in adults, it appears that the immunological memory could play an important role in antibody responses after vaccination [49]. Indeed, in children, in whom we observed no or very low stalk signals, most of the response after vaccination was directed against the globular head domain. This particular 'conserved' trend that was observed in pediatric samples can be attributed to the low age of children (3–9 years) and the possibility for at least some of them of being completely naïve for A/H1N1/California/7/2009 influenza strain at the time of blood draw. The high anti-head antibody signal that was observed after vaccination in children, in contrast to the

low anti-stalk signal, can be explained by different reasons: 1) the head domain of the HA is the most immunogenic part of the HA protein, in contrast to the stalk, which appears to be less immunogenic; 2) antibodies against the stalk domain are generally difficult to be elicited by classical inactivated split and subunit influenza vaccines [50]; and 3) the influenza specific B- (and T-) cells repertoires in young children contain a greater frequency of naïve cells. However, adults have pre-existing populations of influenza-specific memory cells that can target conserved epitopes [51]. This last point seems to validate our results from adult subjects, where we observed a boost in both head and stalk responses after vaccination and a more heterogeneous scenario in comparison to children. In one adult subject, we detected a particularly high anti-stalk response after vaccination and a very low anti-head response, despite a very high HI titer accompanied by increased SRH and NA responses. The unpredictability and complexity of immune responses against influenza vaccination are illustrated by the fact that this subject did not show an increase in the neutralizing antibody response. This peculiar observation can be explained by the interference of a large number of antibodies directed against conserved epitopes of the influenza virus, thus causing the steric hindrance of the hemagglutination activity. This was confirmed by the high SRH titer and the very low MN titer after vaccination, and it supports the superiority of the SRH assay over the HI and MN assays to detect a broader range of functional antibodies. This characteristic may not only reflect the specific nature of the SRH assay, which detects all antibodies directed against various epitopes of HA and NA, it may also reflect the fact that internal influenza virus proteins may be involved in the complement fixation reaction. The small increase in the MN titer, along with the low head response detected with the ELISA, seems to confirm this theory. This particular case supports the strategy that has been adopted in the last few years by some regulatory agencies, such as EMA [45,52], to take into account a combination of different immune responses that are measured by multiple assays for the evaluation of the effectiveness of influenza vaccines. In the present study, we also included the measurement of the NA antibodies in order to broaden the view of the antibody-mediated immune response in both age groups. However, it is important to point out that current licensed influenza vaccines are made with a well standardized amount of HA antigens but not of NA antigens. The understanding of the NA response could become extremely important for the study of the immune response after LAIV administration or natural infection.

5. Conclusions

In conclusion, the competitive ELISA described in here, when supported by parallel assays such as neutralization, SRH, NA inhibition and antibody-dependent cellular cytotoxicity reporter (ADCC), is able to accurately distinguish differences in individual immune responses, thereby allowing the mode of action of different (next-generation) influenza vaccine approaches to be interpreted. Specifically, as reported in several studies [26,53,54], the ADCC assay can reflect the functionality of the antibodies that are detected by ELISA. The results presented here confirm that the classical serological assays that are generally used to evaluate the immunogenicity of HA-based intramuscular/intradermal seasonal influenza vaccines are still valid. However, they could be insufficient in the evaluation of the immune response of next-generation influenza vaccines, especially if used alone.

This preliminary study presents some limitations, mainly based on the small number of samples that were analyzed and the use of HA and head subunits from a single influenza strain. Further studies will be done with the aim to qualify the assay, both by using a mixture of head and stalk reactive monoclonal antibodies as controls and by comparing the results obtained with other assays that are based on the use of chimeric HA proteins to directly detect stalk antibodies. Other parameters will address the influence of the protein concentration that is used during the treatment of samples and the inclusion of conformation-specific monoclonal antibodies to ensure that the head protein retains its native conformation after coating. In this first study, we used a quite high protein concentration in order to make sure that any serum antibodies were fully adsorbed or competed with soluble protein.

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writing—original draft preparation, A.M., A.K.M, C.M.T.; writing—review and editing, O.K., A.T., and E.M.; visualization, A.K.M., A.M.; supervision and project administration, E.M. All authors have read and agreed to the published version of the manuscript.

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Influenza D Virus: Serological Evidence in the Italian Population from 2005 to 2017

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Abstract: Influenza D virus is a novel influenza virus, which was first isolated from an ailing swine in 2011 and later detected in cattle, suggesting that these animals may be a primary natural reservoir. To date, few studies have been performed on human samples and there is no conclusive evidence on the ability of the virus to infect humans. The aim of this serological study was to assess the prevalence of antibodies against influenza D virus in human serum samples collected in Italy from 2005 to 2017. Serum samples were analysed by haemagglutination inhibition and virus neutralization assays. The results showed that the prevalence of antibodies against the virus increased in the human population in Italy from 2005 to 2017, with a trend characterized by a sharp increase in some years, followed by a decline in subsequent years. The virus showed the ability to infect and elicit an immune response in humans. However, prevalence peaks in humans appear to follow epidemics in animals and not to persist in the human population.

Keywords: influenza D virus; seroprevalence; humans; Italy

1. Introduction

Influenza D virus (IDV), a novel influenza virus, was first isolated from an ailing swine in 2011 in Oklahoma, USA [1,2]. Although the viral genome shows approximately 50% overall homology with influenza C virus (ICV), no cross-reactivity with antibodies directed against human ICV and no re-assortment with human ICVs have been observed so far. Moreover, attempts to detect viable recombinant progeny involving ICV and IDV have not been successful [1,3–5].

Several epidemiological and serological studies have reported the isolation of IDV in cattle from many geographic areas (Canada, the United Kingdom, Japan, the United States, Mexico, Luxemburg, Ireland, France and China), suggesting that cattle may be a primary natural reservoir for the virus [6–18]. In addition, two studies have suggested that IDV has circulated among beef cattle since at least 2003/2004 [9,13]. IDV has also been identified in other animal species, such as sheep,

goats, camelids and horses across countries in different continents (Europe, North America, Africa and Asia), but there is no evidence of infections in chickens and turkeys [18–24].

The prevalence of antibodies against IDV in domestic pigs ranges from 9.5% to 11.7% [1,25], indicating that the virus is able to circulate among domestic pigs, but is not yet widespread [26]. A similar IDV antibody prevalence has also been reported in horses in the Midwest United States [22]. A higher seroprevalence (19.1%) has been found in feral pigs, which could have been due to the increased chances of having contact with various domestic and wild animals and, hence, a higher exposure to IDV [27]. Seroprevalence of antibodies against IDV increases substantially especially in newborn calves, as a result of maternal antibodies, and in older cattle. As the level of maternal antibodies declines, calves become more susceptible to IDV infection; this increases the risk of active transmission and the potential to create a virus reservoir [9,12,13,26]. Moreover, IDV can be efficiently transmitted among cattle by direct contact [10]. The presence of IDV in pigs and cattle has also been reported in Italy, where it has been confirmed by PCR and virus isolation, as well as serological analysis for the presence of IDV specific haemagglutination inhibition (HI) antibodies [25,28]. Specifically, a study conducted in the swine population in Northern Italy revealed a high prevalence (11.7%) of antibodies against IDV in 2015, which demonstrated significantly increased seroprevalence compared to the reported rate in 2009 (prevalence 0.6%) [25].

To date, few studies have been performed on human samples. A screening study of human serum samples showed that 1.3% of the general population had antibody titres against IDV [1], while in Scotland no evidence of IDV infection emerged from the analysis of archived respiratory samples [29]. However, a serological study conducted in Florida reported a seroprevalence of 94% among workers exposed to cattle (32/35 samples) [30].

Although there is no conclusive evidence that IDV can infect humans, a study conducted in ferrets, which are the preferred human surrogate animal models for influenza virus studies, has shown that the virus is able to spread among ferrets and that it has a broader cellular tropism than human ICVs [1]. These features indicate that IDV carries the risk of becoming a potential threat to public health.

The aim of this serological study was to assess the prevalence of antibodies against IDV in archived human serum samples collected in Italy from 2005 to 2017.

2. Materials and Methods

2.1. Influenza Viruses

Influenza D/bovine/Oklahoma/660/2013 virus was originally isolated from the bovine herds of Oklahoma.

Influenza C/Victoria/2/2012 virus was originally isolated in 2012 from a nasopharyngeal swab of a child with clinical symptoms of acute respiratory infection and was submitted for virus isolation to the Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia. The virus was isolated after cultivation in Madin Darby Canine Kidney (MDCK) cells at 33 °C, together with RT-PCR diagnosis and sequence confirmation.

IDV and ICV were propagated in MDCK cells, using UltraMDCK serum-free medium (SFM) supplemented with 2 μg/mL of acetylated trypsin (IDV) and trypsin (ICV) from bovine pancreas (Sigma-Aldrich, Saint Louis, MO, USA) and 100 IU/mL penicillin-streptomycin.

Cells were seeded in a T175 cm^2 culture flask at a density of 1×10^6 cells/mL with UltraMDCK SFM. After 18–20 h, the cell monolayer was washed twice with sterile Dulbecco's phosphate buffered saline (DPBS). After the DPBS had been carefully removed, cells were infected with 3.5 mL (IDV) or 10mL (ICV) of UltraMDCK SFM (without Trypsin) containing the respective virus at a multiplicity of infection of 0.001. After 1 h of incubation at 33 °C in a humidified atmosphere with 5% CO_2, 50 mL of UltraMDCK SFM containing a final concentration of 2 μg of trypsin acetylated (IDV) and 0.5 μg of TPCK (ICV) was added to the flask. The infected cells were incubated at 33 °C in a humidified atmosphere with 5% CO_2 for 36 h. At the end of the incubation period, additional acetylated trypsin

was added up to a final concentration of 1 µg/mL and the flask was incubated at 37 °C in a humidified atmosphere with 5% CO_2 for another 36–48 h. The cytopathic effect (CPE) was monitored every day, along with the hemagglutination (HA) titre of the supernatant. At 90% of the CPE, the culture medium was harvested, centrifuged at 4 °C in order to remove the cell debris, and stored at −80 °C.

2.2. Serum Samples

Archived human serum samples from adults (≥18 years old) were obtained from the Serum Bank of the Laboratory of Molecular Epidemiology, Department of Molecular and Developmental Medicine, University of Siena, Siena, Italy.

The samples were anonymously collected in Tuscany (Central Italy) and Apulia (Southern Italy) and stored in compliance with Italian ethics law. The only information available was the age of each subject and the year of sampling. A total of 1281 serum samples collected from 2005 to 2017 (approximately 100 serum samples for each year) were randomly selected, though a balanced distribution between males and females and among age-groups in each year was ensured, according to the availability of serum samples for each year (Table S1).

Influenza D (swine) and C (rooster) hyperimmune serum samples, kindly provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna (IZSLER, Brescia, Italy) and Institut National de la Recherche Agronomique (INRA) (France) and Ecole Nationale Vétérinaire de Toulouse (INP-ENVT) (France), were used as positive controls.

Human serum without IgA, IgM and IgG was used as a negative control (Sigma-Aldrich, S5393).

2.3. Haemagglutination Inhibition Assay

The HI assay was performed as described in Hause et al. [1]. All serum samples, including positive and negative controls, were pre-treated with receptor-destroying enzyme from Vibrio Cholerae (Sigma Aldrich, Milano, Italy) (ratio 1:5) followed by heat inactivation for 1 h at 56 °C. Serum samples were tested in duplicate by using turkey red blood cells (0.35%). The antibody titre was expressed as the reciprocal of the highest serum dilution that showed complete inhibition of agglutination. Since the starting dilution was 1:10, when the titre was below the detectable threshold, the results were conventionally expressed as 5 for calculation purposes [31].

2.4. Virus Neutralization Assay

The MDCK cell cultures were grown at 37 °C in 5% CO_2 and pre-incubated in a 96-well plate for 4 h.

Serum samples, including positive and negative controls, previously heat-inactivated at 56 °C for 30 min and tested in duplicate, were two-fold diluted with EMEM culture medium supplemented with 0.5% fetal bovine serum in a 96-well plate and mixed with an equal volume of virus (100 TCID50/well). After 1 h incubation at 37 °C in 5% CO_2, the mixture was added to the MDCK cell suspension (1.5×10^5 cells/mL). Plates were read for HA activity in the supernatant after three days of incubation at 37 °C in 5% CO_2.

2.5. Statistical Analysis

According to the definition of seropositivity used for other newly emerging viruses, all positive serological responses (HI titres ≥1:10) were classified as seropositive, while non-detectable HI responses (<1:10) were regarded as negative and arbitrarily expressed as a value of 5 [31]. In addition, positive titres were classified in positive (≥1:10, ≥1:20), and highly positive (≥1:40). For the purpose of direct comparison of HI and virus neutralization (VN) assays, the proportions for each category of titres were calculated by applying the total number of serum samples tested in the HI and VN assays. Calculation of the 95% confidence intervals for the proportions was based on the Clopper–Pearson exact method [32].

For positive and highly positive titres, the Pearson's Chi-squared test was used to verify the significant differences among the proportions of titres between the two assays across years, and to make yearly-based comparisons of the observed proportions for each assay. The Marascuilo procedure was used for the post-hoc analysis, which accounts for multiple comparisons. Relative changes of proportions were evaluated in relation to the value of the proportion measured for 2005, the first year tested, which was used as the base year for the analysis. The Hodrick–Prescott filter, with a smoothness penalty parameter $\lambda = 1600$, was implemented over the time series of the relative changes in proportions in order to estimate their trend components [33]. All the analyses were made as two-sided tests and conducted at a significance level of 5%. RStudio (version 1.1.463) was used for all the statistical analyses.

3. Results

3.1. Investigations on Potential Cross-Reactivity between IDV- and ICV-Positive Serum Samples

The specificity of the HI assay with respect to the potential cross-reactivity between IDV and ICV was evaluated by testing both viral antigens against IDV and ICV specific hyper-immune antisera generated in swine and rooster. As shown in Table 1, no cross-reaction between IDV and ICV was observed. The anti-serum specific for the D/bovine/Oklahoma/660/2013 strain showed a high HI titre (1:10,240) as did the anti-serum specific for the C/Victoria/2/2012 (HI titre 1:640). No HI titres were detectable when the hyper-immune antisera were tested against the respective heterologous influenza C or D strain. These results demonstrated the specificity of the IDV HI assay and were therefore used for the analysis of the presence of IDV-specific antibodies in human serum samples.

Table 1. HI cross-reactivity between IDV and ICV against hyper immune sera.

Influenza Viruses	IDV Antiserum HI Titre	ICV Antiserum HI Titre
D/bovine/Oklahoma/660/2013	1:10,240	5
C/Victoria/2/2012	5	1:640

The IDV hyperimmune serum sample was used as positive control in HI and VN assays showing a titre range of 2560–10,240 and 1280–5120, respectively.

3.2. Analysis of Human Serum Samples for the Presence of IDV-Specific Antibodies

A total of 1281 human serum samples, collected randomly from adults in the Italian regions of Tuscany and Apulia from 2005 to 2017, were tested by HI assay in order to detect the presence of antibodies against IDV. The results clearly show that IDV specific HI antibodies were present in at least a small subset of serum samples taken in every single year between 2005 and 2017, although IDV was isolated and described for the first time in 2011 ((A) in Table 2). IDV antibodies displayed low levels, between 5.1% and 9.8%, in the years 2005–2007, followed by a sharp increase in 2008; the highest levels (33.9–46.0%) were reached in 2008, 2009, 2010, 2013, 2014 and 2016, while the lowest levels (11.9–25.7%) were seen in 2011, 2012, 2015 and 2017.

In addition, the highest levels of HI seropositivity (HI titres ≥ 1:40) were found in serum samples collected in 2008, 2009, 2012, 2013, 2014 and 2016. The human sera with positive HI titres (≥1:10) were then tested in an IDV specific VN assay to confirm the positive HI titres and subsequently the specificity of the HI assay for IDV ((B) in Table 2).

3.3. Pearson's Chi-Squared Test for Multi-Proportions

Differences between the HI and VN assays proportions in homologous class of titres were not significant. By contrast, the analysis of proportions among years showed significant differences for both assays in each class of titres (Table S2). Comparisons of the titre proportions over years were conducted on each class of titres except the negative class, as this class is overtly complementary to

the positive (≥1:10) class of titres (Table S3). In the HI assay, the proportions of titres ≥1:10 in the first three years (2005–2007) proved to be almost always lower than the values measured in the other years. The proportion observed in the year 2017 significantly differed only when compared with the values recorded for the years 2010–2014.

Likewise, in the VN assay, the proportions in the years 2005 and 2006 proved to be almost always different from the proportions in the other years. The 2007 value, however, was significantly different only when compared with the values for the years 2013–2014, as well as the 2017 value. Significant differences in the proportions of HI titres ≥1:20 were identified only between the peak values (in 2008, 2009, 2013, 2014) and the values recorded in the first two years, 2005 and 2006. Regarding the class of highly positive titres (≥1:40), there was not enough evidence to identify significance on pairwise comparisons of proportions.

Our analysis of the relative changes in proportions provided insights into the dynamics of the proportions of titres. Figure 1 shows the normalized proportions of negative, positive and highly positive HI titres and their corresponding trend curves.

Table 2. IDV specific HI (A) and VN (B) titres of human serum samples collected from 2005 to 2017 in Italy.

HI Assay															
Year		**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**Total**
Samples		99	101	82	95	101	83	101	102	100	100	101	115	101	1281
Titre	5	94	93	74	59	61	47	83	82	59	54	75	76	89	**946**
	≥1:10	5	8	8	36	40	36	18	20	41	46	26	39	12	**335**
	≥1:20	4	8	1	31	27	15	14	17	31	30	19	27	12	**236**
	≥1:40	3	4	0	15	14	6	6	12	17	12	9	13	8	**119**
	≥1:80	2	1	0	7	9	3	2	8	7	2	2	4	5	**52**
	≥1:160	1	0	0	5	2	0	2	0	2	0	0	3	3	**18**
N≥1:10		5	8	8	36	40	36	18	20	41	46	26	39	12	**335**
%≥1:10		5.1%	7.9%	9.8%	37.9%	39.6%	43.4%	17.8%	19.6%	41.0%	46.0%	25.7%	33.9%	11.9%	**26.2%**
CI 95% lower		1.7%	3.5%	4.3%	28.1%	30.0%	32.5%	10.9%	12.4%	31.3%	36.0%	17.6%	25.3%	6.3%	**23.8%**
CI 95% upper		11.4%	15.0%	18.3%	48.4%	49.8%	54.7%	26.7%	28.6%	51.3%	56.3%	35.4%	43.3%	19.8%	**28.6%**

(**A**)

VN Assay															
Year		**2005**	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**Total**
Samples		5	8	8	36	40	36	18	20	41	46	26	39	12	335
Titre	5	0	1	0	2	7	16	1	5	2	4	4	9	4	**55**
	≥1:10	5	7	8	34	33	20	17	15	39	42	22	30	8	**280**
	≥1:20	5	5	8	33	18	17	15	13	29	35	35	20	7	**240**
	≥1:40	4	4	2	10	2	5	9	9	12	14	14	7	4	**96**
	≥1:80	1	1	0	1	0	1	2	0	4	0	0	0	1	**11**
	≥1:160	0	0	0	1	0	0	2	0	1	0	0	0	0	**4**
N≥1:10		5	7	8	34	33	20	17	15	39	42	22	30	8	**280**
%≥1:10		5.1%	6.9%	9.8%	35.8%	32.7%	24.1%	16.8%	14.7%	39.0%	42.0%	21.8%	26.1%	7.9%	**21.9%**
CI 95% lower		1.7%	2.8%	4.3%	26.2%	23.7%	15.4%	10.1%	8.5%	29.4%	32.2%	14.2%	18.3%	3.5%	**19.6%**
CI 95% upper		11.4%	13.8%	18.3%	46.3%	42.7%	34.7%	25.6%	23.1%	49.3%	52.3%	31.1%	35.1%	15.0%	**24.2%**

(**B**)

Over the years, the proportion of the negative HI titres was always lower than the base value of 2005 (94.9%). The positive titres showed similar bimodal patterns, with relative minima in 2011, 2015 and 2017. The proportion of the positive titres (≥1:10) peaked in 2010 and 2014. The proportions of positive titres ≥1:20 and ≥1:40 showed an absolute minimum in 2007 and an upsurge in 2008 and 2013, a trend that differed slightly from that of the ≥1:10 positive titres.

The curves of the normalized proportions of the titres, as well as their trend curves, displayed similar characteristics in the HI and VN assays over the period 2005–2017 (Figure S1).

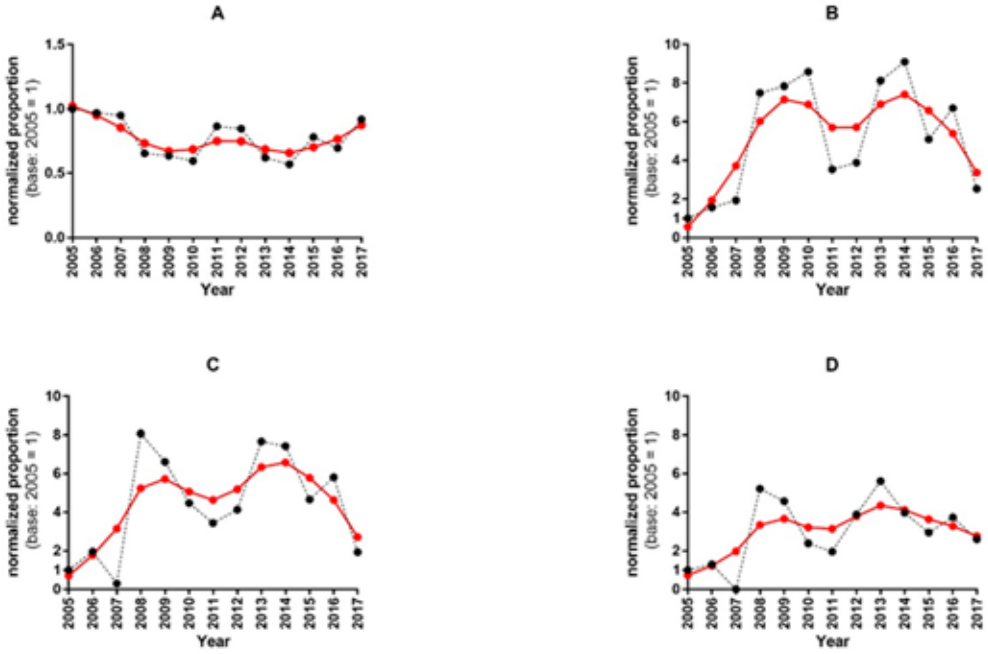

Figure 1. Time course of the normalized proportions and their trend lines in HI titres. The panels (**A**–**D**) display the normalized proportions (black dots) of negative (5), positive (≥1:10, ≥1:20), and high positive (≥1:40) HI titres respectively, with the trend curves (red dots) estimated by using the Hodrick–Prescott filter. Panel (**A**): the dynamics of the time series shows values that are almost always lower than the base 2005 values. After 2014, the trend seems to have turned upwards towards the initial base level. Panel (**B**): the time series of the positive titres (≥10) shows a bimodal pattern, with peaks in 2010 and 2014, preceded by huge upsurges in 2008 and 2013. Peaks in the trend curve were observed in 2009 and 2014. Panel (**C**): the positive titres ≥20 show a similar bimodal pattern in the time series, with peaks in normalized proportions occurring in 2008 and 2013. The trend curve shows two peaks in the same years as that of the positive titres ≥10. Panel (**D**): the time series of the normalized proportions of highly positive titres (≥1:40) displays its highest values in 2008 and 2013. In the trend curve, the first peak is delayed by one year (2009), and the second peak coincides with the year 2013.

4. Discussion

The detection and isolation of IDV in pigs with influenza-like clinical signs and respiratory distress in 2011, and the evidence of a seroprevalence of 1.3% of humans [1] in combination with the circulation of IDV in pigs in Northern Italy which was confirmed in 2015 [25], have raised the question of whether IDV antibodies can be found in the Italian population since this potential outbreak. This hypothesis is supported by a study conducted in Florida by White et al., which reported a seroprevalence of 94% among workers exposed to cattle [30].

The results of the present study indicate that the findings of studies conducted in various countries also apply to Italy. The low seroprevalence (5.1%) of IDV specific antibodies found in the human serum samples from the first year of the study (2005) suggests that IDV may have circulated at least in Italy already before 2005. This finding is supported by a study by Luo et al. [13], which suggested that Nebraska beef cattle had been exposed to IDV since at least 2003, and that the virus may have already circulated at least 8 years before its detection.

The potential IDV circulation in pigs in Northern Italy in 2015 [25], which was confirmed by PCR analysis, positive virus isolation, and increased IDV specific HI antibody responses in animals from the farms affected, may have been associated with an increase in IDV HI-specific antibodies in humans from 25.7% in 2015 to 33.9% in 2016. These data also indicate that there could have been undetected IDV circulation in pigs and/or cattle already in 2007 and reflect a confirmed outbreak in

cattle in 2011 [7], since the seroprevalence of HI specific antibodies in humans increased from 9.8% in 2007 to 37.9% in 2008 and from 19.6% in 2012 to 41.0% in 2013.

These data clearly show that seroprevalence against IDV increased in Italy from 2005 to 2017. This increase was not constant over the years, it shows sharp rises in some years followed by drops in subsequent years. The seroprevalence peaks detected in humans appear to follow IDV epidemics in animals, as an epidemic in cattle in France in 2011 [7] and an outbreak in pigs in Italy in 2015 [25] have been reported. Moreover, as the titres did not remain high in the years following the increase, but dropped to lower levels, we could speculate that a spill over event from an animal reservoir occurred, and that the virus does not circulate primarily in humans. However, IDV proved able to elicit an immune response in humans. The background seropositivity rate in the population was higher in 2017 (11.9%) than in 2005 (5.1%), although this difference did not prove statistically significant. Follow-up studies over the next couples of years should be performed in order to determine whether the titres drop further to levels below 10% over the years or remain at a higher level than that observed in 2005. In addition, cattle and pigs should be carefully observed and analysed with respect to potential new outbreaks, which could be followed by an increase in IDV-specific antibodies in humans in the subsequent year.

Although IDV was first isolated from a diseased pig in 2011 [1], this virus is notable for being the first influenza virus identified in cattle [3]. This is supported by evidences of past infection in cattle from the same farm as the diseased pig. Finally, additional studies revealed cows to be the primary reservoir [3,9–12]. Since then, IDV has been isolated from cattle and pigs in several countries, including China, the United States and France [3–8], and antibodies against the virus have been found in sheep, goats, horses and camelids in China, Ethiopia, France, Japan, Mongolia, Ireland and the United States [19–24].

The origin and ecology of IDV remain unknown. Although it is not yet known how or when IDV first emerged, analysis of archived serum samples by SJCEIRS (St. Jude Children's Research Hospital Center of Excellence for Influenza Research and Surveillance) suggests that IDV has been circulating in cattle since at least 2004 [2,9,10,13]. IDV infections in cattle also tend to be associated with other respiratory infections, particularly pneumonia, but the significance of this observation is not known [8].

To date, no indications that IDV can cause disease in humans have been found, but several groups have already signalled its potential threat as an emerging pathogen in specific target groups, such as cattle-workers [5], or as a considerable public health risk [2,5,13,26,34,35]. This eventuality is supported by a number of findings by different groups. One of the main risks stems from the ability of the virus to infect and to be transmitted to a number of domestic mammal species, such as cattle, pigs, goats, sheep and camelids, and also to wild animals, such as feral pigs [22]. In addition, it has been shown that IDV can infect ferrets, the gold standard for influenza studies in animals [1,26], and guinea pigs, as shown by transmission studies [26,36].

Seroprevalence studies in humans have shown that the risk of transmission from infected cattle to humans may be very high. Indeed, seroprevalence rates of 91% (HI assay) and 97% (VN assay) have been documented in cattle-workers, as opposed to 18% (on VN assay) in control subjects without contact with cattle [26,30]. The results of the present study show seroprevalence peaks in humans that seem to follow IDV epidemics in animals (outbreak in pigs in Italy in 2015 [25]). However, further studies on the circulation of IDV in animals in the same years would be useful to support this hypothesis. The increased risk of transmission to humans is supported by the finding that the IDV hemagglutinin-esterase-fusion glycoprotein exhibits an open receptor-binding cavity, which forms the basis for its broad cell tropism and, consequently, its broad host tropism [37]. A valuable tool for studies on IDV replication kinetics and cell tropism has been provided by Holwerda et al. [38], who used "primary well-differentiated human airway epithelial cells" as an in vitro respiratory epithelium model of humans.

At least three antigenic lineages of IDV have been identified—D/Oklahoma, D/660 and D/Japan—and HI analysis have shown up to a 10-fold loss in cross-reactivity against heterologous

antiserum [8,39,40]. Serological analysis in this study has been conducted using D/660-like strain; however, the use of a single IDV strain may ultimately lead to an underestimation of the true seroprevalence in Italian population, leaving the conclusions of this study unchanged.

Despite the solid results yielded by a considerable number of studies, our current knowledge of IDV is still limited, and neither potential threats to exposed individuals nor public health issues can be fully excluded. Consequently, additional research on IDV and diligent observation of IDV prevalence in the various animal hosts and in potentially affected individuals should be conducted in the future.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/1/30/s1, Figure S1: VN vs. HI, normalized proportions and trend lines for the titre class ≥1:10, Table S1: Serum samples collected in Italy from 2005 to 2017, Table S2: Results of the multiple proportion test among years, Table S3: Multiple comparisons of proportions of positive titres (≥1:10, ≥1:20) for HI and VN assays along the period 2005–2017.

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NON-COMMISSIONED: REVIEW, EXPERT COMMENTARY, MEETING REPORT

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How to assess the effectiveness of nasal influenza vaccines? Role and measurement of sIgA in mucosal secretions

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Abstract

Secretory IgAs (sIgA) constitute the principal isotype of antibodies present in nasal and mucosal secretions. They are secreted by plasma cells adjacent to the mucosal epithelial cells, the site where infection occurs, and are the main humoral mediator of mucosal immunity. Mucosally delivered vaccines, such as live attenuated influenza vaccine (LAIV), are able to mimic natural infection without causing disease or virus transmission and mainly elicit a local immune response. The measurement of sIgA concentrations in nasal swab/wash and saliva samples is therefore a valuable tool for evaluating their role in the effectiveness of such vaccines. Here, we describe two standardized assays (enzyme-linked immunosorbent assay and microneutralization) available for the quantification of sIgA and discuss the advantages and limitations of their use.

KEYWORDS

enzyme-linked immunosorbent assay, influenza vaccines, influenza virus, mucosal immunity, secretory IgAs

1 | INTRODUCTION

Influenza is a disease with high morbidity and mortality which is caused by influenza viruses of types A and B.[1,2] Seasonal influenza epidemics are estimated to result in 3-5 million cases of severe illness and about 290 000-650 000 respiratory deaths worldwide.[2]

2 | SECRETORY IGA PRODUCTION UPON NATURAL INFLUENZA INFECTION

Influenza viruses infect humans through the mucosal epithelium covering the upper respiratory tract; thus, the respiratory epithelium constitutes the site of virus entry, infection, and host immune response (Figure 1). Antibodies located on the surface of the mucosa represent the major immune components providing protection against influenza. Upon infection, the human humoral immune response is activated leading principally to the production of local secretory IgA (sIgA) in the mucosa of the upper respiratory system, serum IgA and IgG antibodies.[3]

2.1 | Role of IgA and IgG antibodies

Whereas sIgA antibodies have the role to neutralize potential pathogens at the entrance site before they could attach epithelial cells and overcome the epithelium surface, serum IgA represent

Elena Gianchecchi, Alessandro Manenti and Emanuele Montomoli contributed equal to this work.

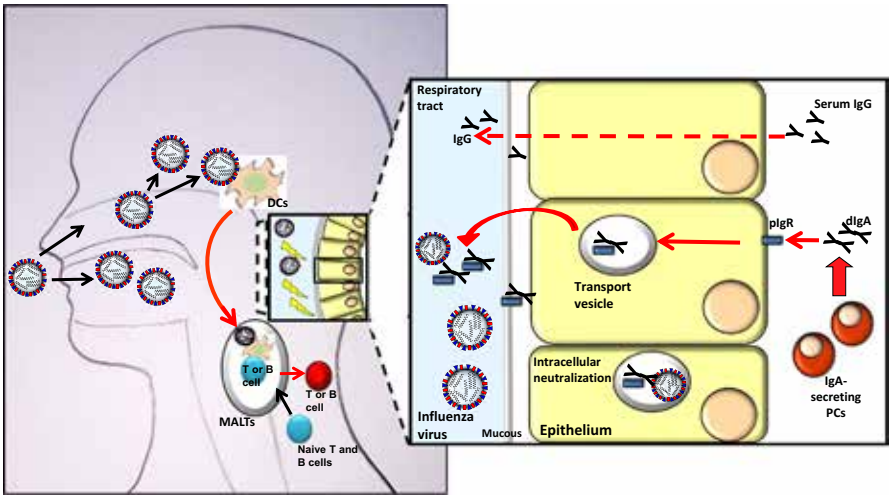

FIGURE 1 Simplified scheme of immune responses following influenza virus infection of the upper respiratory tract with focus on induction and mode of action of secreted IgA (sIgA). Abbreviations: B cell, B lymphocyte; DCs, dendritic cells; dIgA, dimeric IgA; IgA, immunoglobulin A; IgG, immunoglobulin G; MALT, mucosa-associated lymphoid tissue; PCs, plasma cells; pIgR, polymeric immunoglobulin receptor (pIgR); T cell, T lymphocyte. Influenza viruses infect epithelial cells of the mucosa and induce mucosal immune responses. Mucosal immune system consists of two sites. Inductive site (MALT) for antigen uptake by DCs and priming of T and B cells for IgA antibody production. Effector site with IgA-secreting PCs. DCs take up exogenous virus antigens (from virus particles or apoptotic infected cells) by endocytosis and activate naïve T and B cells. PCs secrete IgA antibodies. IgG antibodies transude from the serum to the mucus by diffusion and provide protection against homologous influenza viruses. dIgA are actively transcytosed across epithelial cells via pIgR and provide protection against homologous and heterologous influenza viruses. dIgA can bind to newly synthesized viral proteins within virus-infected epithelial cells and prevent virus

"backup" antibodies whose function is to respond in case of systemic infection due to invasion across the mucosal epithelium.[4] IgG-secreting cells are produced in the mucosa-associated lymphoid tissues (MALTs) and regional lymph nodes. IgG antibodies are secreted in the bloodstream and, reached the mucosal tissues, move via diffusion from the serum to the mucus.[5] IgA is characterized by an elevated ability in preventing virus infection, whereas IgG exerts only a marginal role in providing protection toward infections affecting the upper respiratory system. However, IgG antibodies are principally involved in reducing viral pneumonia.[6]

2.2 | Role of cellular immune responses

Along with the humoral immunity, also the cell-mediated immune (CMI) response is activated after influenza infection. Unlike humoral response, capable of neutralizing activity, CMI is able to prevent virus replication and decrease the time for recovery.[7,8] $CD4^+$ follicular helper T (Th) lymphocytes in presence of antigen-presenting cells (APCs), such as dendritic cells (DCs) and influenza antigens, induce the differentiation of naïve B cells into IgA-secreting plasma cells (PCs). sIgA constitute the principal isotype of antibodies present in external secretions, such as nasal fluid, saliva, milk, colostrum intestinal fluid, and gallbladder bile.[9] In the upper respiratory tract, sIgA antibodies are secreted by mucosal PCs adjacent to the mucosal epithelial layer at the site of infection[10] and represent the main humoral mediator of nasal immunity.[11]

2.3 | Immune mechanisms contributing to disease reduction or protection

In influenza-naive subjects, the clearance of primary viral infection occurs through sIgA and cytotoxic T lymphocytes (CTLs). More specifically, sIgA appear on day 5 post-infection, and their level rapidly increases in the nasal wash until day 7-10 post-infection, when it reaches a plateau. IgA local immune response persists for a period of 3-5 months[5,12,13] and then gradually diminishes returning to the pre-immunization levels within 6 months.[14] In addition, it is possible to detect IgA-producing memory cells locally.[5,12,13] CTLs appear transiently in the nasal mucosa and peak on day 7 after infection. sIgA have a pivotal role in protecting against influenza infection of the upper respiratory mucosal surfaces, since they can disarm the virus either before it crosses the mucosal barrier[15] or in infected epithelial cells by intracellular neutralization.[15,16] The magnitude of the IgA antibody response is directly correlated with resistance to new infections.[17] In addition, IgA is the predominant Ig isotype in local secretions after secondary infection and an IgA response is also detected in the serum upon subsequent infection which support its additional important role in protection against influenza virus re-infections.[18]

Along with IgA, IgM antibodies are also secreted actively across the mucosa and may contribute to protection by preventing viral entry into the cells and also interfering with virus replication in the cells.[5,18] The potential protective role of IgM antibodies is supported by a study in mice which has shown that IgM antibodies can

neutralize influenza viruses in the presence of complement just as well as IgG antibodies.[19]

2.4 | IgA immune responses upon influenza virus infection of the mucosa of the upper respiratory tract

On the basolateral surface of the epithelial cells in the lamina propria of mucosal tissue, a polymeric Ig receptor (pIgR) links the dimeric IgA (dIgA) and moves to the apical side (Figure 1). During the process of transcytosis across the epithelial cells, polymeric IgA (pIgA) acquires the secretory component (SC), producing sIgA. Secretory component is an unusual extra polypeptide that constitutes the extracellular portion of pIgR upon cleavage by a selective protease.[20] The presence of SC provides sIgA a greater functional stability, both by masking the protease sites from proteolytic degradation operated by proteases present in mucosal secretions[21] and by sustaining the association of monomeric IgA (mIgA).[22]

IgA do not promote the activation of the inflammatory complement system, a feature which is critical to maintaining the integrity of the mucosal barrier.[23]

2.5 | Presentation forms and functions of IgA antibodies

In human serum, IgA are mainly present in the monomeric form with two α-heavy and two light chains. On the other hand, in external secretions IgA are highly heterogeneous in terms of their quaternary structure but the majority are in polymeric form.[24] sIgA are generally present as a dimer, despite, and at low frequency, as larger polymeric forms (pIgA) especially tetramers.[25]

It has been hypothesized that pIgA may have a higher ability than mIgA to neutralize intracellular viral particle assembly by binding newly synthesized viral proteins.[5,17,26] It has also been demonstrated that the polymeric nature of sIgA was responsible for their elevated cross-reactivity, thereby increasing the avidity of this antibody subclass in comparison with mIgA and serum IgG.[27,28]

The best neutralizing activity and the higher avidity of human pIgA than mIgA can be attributed to the presence of multiple antigen-binding sites located on each Ig polymer, indicating that the quaternary structure plays a key role for their potency.[25] This result is in accordance with previous researches conducted on mice.[27,29,30] A recent study by Saito and colleagues demonstrated that IgA tetramerization improves target breadth exerting no effect on potency of functionality of anti-influenza virus broadly neutralizing antibody.[31] The higher anti-viral activity of pIgA than mIgA is particularly important, considering the anatomical site of sIgA action.[10] pIgA appears to have a greater inhibitory potential in preventing viral attachment and virus neutralization than mIgA and also IgG.[29,32,33] Another study showed the existence of larger pIgA in addition to tetrameric sIgA in the upper respiratory tract. The proportion of this polymeric form is approximately 20% of the total IgA.[34]

In summary, the mucosal surface is endowed with two protective barriers against viral infection, both of which involve mucosal IgA, that is, extracellular sIgA and intracellular pIgA.[29]

3 | IGA IMMUNE RESPONSE UPON INFLUENZA VACCINATION

Conventional inactivated influenza vaccines (IIVs), generally delivered through subcutaneous or intramuscular injection, are today still the most efficient, valuable, and low-cost tools to effectively reduce influenza virus infections and subsequently morbidity and mortality.[35] This parental administration is able to increase the serum antibody level in the systemic immune compartment, but it is not able to trigger a local mucosal immune response at the site of primary infection, that is, an induction of sIgA which exhibit a wide cross-protection activity. This represents a limit for conventional inactivated influenza vaccines in conferring full protection against infection.[36]

While natural infection is able to induce both mucosal and systemic heterosubtypic responses, the immunity induced by parenterally application of inactivated influenza vaccines is generally virus subtype-specific.[37] In pre-immunized subjects, the natural contact with the pathogen causes a rapid synthesis of IgA and IgG by B memory cells already 3 days after infection. These immunoglobulins form Ig-virus complexes which result in virus inactivation.[17]

In recent years, an increasing number of pre-clinical and clinical studies have been performed which have led to a better understanding how mucosal antibodies could be elicited by intranasal vaccination with live attenuated influenza vaccines (LAIV). Live attenuated influenza vaccines is administered as a nasal spray and contains a cold-adapted (ca) live attenuated influenza virus which, in contrast to wild-type viruses, is able to replicate well at lower temperatures (around 25°C) and as such only in the upper respiratory tract, but not at higher temperatures (37°C) which does not allow replication in the lower respiratory tract including the lungs. Such property does not allow the ca virus to replicate in lung tissues or cause the onset of influenza-like illness.[38] LAIVs have been introduced firstly by Russia.[39] They have been used in adults since the 1950s, and from 1987 onwards, the use of Russian LAIV for the prophylaxis of influenza has been widely extended to all age groups including children aged over 3 years.[40] LAIVs have been licensed in the Unites States (US) in 2003 for healthy subjects aged 2-49 years and in the European Union (EU) in 2012 for healthy children aged 2-17 years.[41] Whereas some countries, including Russia, have licensed only trivalent LAIVs (T-LAIVs), recently a quadrivalent LAIV (Q-LAIV) vaccine (MedImmune/AstraZeneca) has been introduced in other countries, such as the US (since 2012), Canada (since 2013), and EU (since 2015), under the trade names FluMist™ in the US and Canada, and Fluenz^{R} in the EU.[42]

Studies performed in mice have demonstrated the predominant protective role played by sIgA,[43,44] even in case of absence of T cells.[45] Specifically, the passive intranasal transfer of anti-influenza A IgA from the respiratory tract of mice immunized with live

influenza virus has been seen to provide protection in naive mice.[43] Accordingly, this protection was suppressed by the intranasal instillation of anti-IgA,[46] whereas it was not affected by treatment with anti-IgM or anti-IgG antibodies. This result supports the importance of IgA as a mediator of murine nasal anti-influenza virus immunity in immunocompetent mice.[47]

Furthermore, several studies have found a higher level of correlation between the degree of protection and the antibody secretory level than serum antibodies both in mice[48] and in humans.[5]

T-LAIVs have been widely investigated in several clinical studies conducted on different age cohorts throughout the world.[49] After influenza LAIV administration, as well as natural infection, sIgA are produced by memory B lymphocytes.

The immunogenicity, efficacy, or effectiveness of LAIVs in comparison with IIVs has been analyzed in a number of studies.[50-54] A meta-analysis of a number of investigations has shown that the LAIV has been less effective than IIV in general.[55] However, individual studies with respect to mismatched vaccine strains or in children with underlying diseases such as asthma have shown a higher efficiency in comparison to IIV, for example, with respect to a circulating variant (A/Sydney/H3N2) not present in the vaccine composition with an efficacy of 86% against this mismatched circulating strain[51] or in children affected by asthma[53] or recurrent respiratory tract infections.[52]

A more recent study conducted by McLean[54] reported a similar effectiveness provided by a Q-LAIV and IIV against a new antigenic A(H3N2) variant, whereas a considerable higher protection was provided by Q-LAIV compared to IIV toward a drifted influenza B strain.

Data obtained from the US reported an apparent lack of LAIV effectiveness in the 2015/2016 influenza season, especially toward A/H1N1 vaccine component. Such results have led the Advisory Committee on Immunization Practices (ACIP) not to use the LAIV in the US during the 2016/2017 seasons.[56] Conversely from the observations reported in the US, a higher overall protection of the LAIV against laboratory-confirmed infection with the A/H1N1 strain in comparison with IIV has been reported by the UK[57] and Finland in the 2015/2016 season.[58] For these reasons, LAIV use is closely monitored, but it is still recommended in these countries as well as in Norway, although the motives underlying this difference have not been elucidated yet.

The persistence of protective mucosal immune responses upon LAIV immunization has been investigated in several studies. Murphy and Clements[45] found elevated levels of IgA that recognize HA, and reduced levels of IgM and IgG, in nasal washes obtained from naïve children infected 2 weeks earlier by means of attenuated A viruses. In about 50% of the vaccinees, IgA and IgG in the nasal wash persisted for 1 year. Subsequent studies confirmed the persistence of long-term (at least 1 year) immunological memory following LAIV vaccination[59] and of serum IgG.[6] The longevity of local immune response up to a year indicates that the mucosal immune system is well developed also in young children.

Clinical Studies have shown that protection after LAIV vaccination is correlated with local anti-hemagglutinin (HA) IgA and anti-neuraminidase antibodies in serum,[6] whereas IgG antibodies are the main effectors in providing protection in the mucosal compartments of human vaccinated with inactivated vaccines. These antibodies derive from plasma through a process of passive transudation following a concentration gradient between plasma and nasal IgG.[60] The substantial differences in the presence of anti-viral IgA and IgG antibodies in nasal washes or serum of individuals vaccinated either with LAIV or with IIV indicate that these two vaccines are inducing fundamentally different immune responses resulting in different mechanisms of protection. Whereas the protection of the upper respiratory tract is provided mainly by IgA with IgG playing a minor part, the latter play a pivotal role in the protection of the lungs.[6,60-62] These differences in the specific immune responses have been confirmed by the recent meta-analysis of the group of Wen et al[62] which identified 191 and 195 differentially expressed genes in IIV and LAIV recipients, respectively. Whereas IIV induced the up-regulation of genes associated with both the innate immune response and the humoral immune response, LAIV mostly elicited the innate immunity.

These data suggest that intranasal vaccinations may be the best choice to achieve immune responses which mimic natural infections by stimulating both systemic and mucosal immune response,[63,64] but without causing the signs and symptoms associated with influenza illness.[49] Nowadays, intranasal vaccination against influenza is mainly made up through ca LAIVs.

However, other alternative ways used to induce mucosal immunity are currently available. These include the following: intranasal vaccinations using inactivated whole or split influenza vaccines,[65,66] sublingual administration of adjuvanted influenza vaccines,[67] and novel types of LAIVs, for example, formulated by depleting the *NS1* gene. *NS1* encodes for a non-structural protein, resulting in attenuated viruses (DelNS1 viruses) unable to overcome the anti-viral defenses of infected cells.[37,68]

4 | ELISA ASSAYS FOR THE DETERMINATION OF THE IGA CONTENT IN TEST SAMPLES

According to the type of influenza vaccine used and the route of administration, specific compartments of the human immune system are stimulated. A local mucosal immune response is elicited by natural infection or intranasal vaccination, while a systemic immune response develops after parenteral vaccination.[69] As a consequence of stimulating different components of the immune system via mucosal or parenteral application of vaccines, the induction of serum hemagglutinin inhibition (HI) antibody titers, which are still considered as the gold standard in assessing influenza vaccine immunogenicity, is generally lower after intranasal vaccination than those elicited by intramuscular vaccination[50]; conversely, high levels of nasal IgA have been observed in recipients of LAIV.[63] Of out a couple of laboratory tests that can be used to assess influenza antibody levels, the enzyme-linked

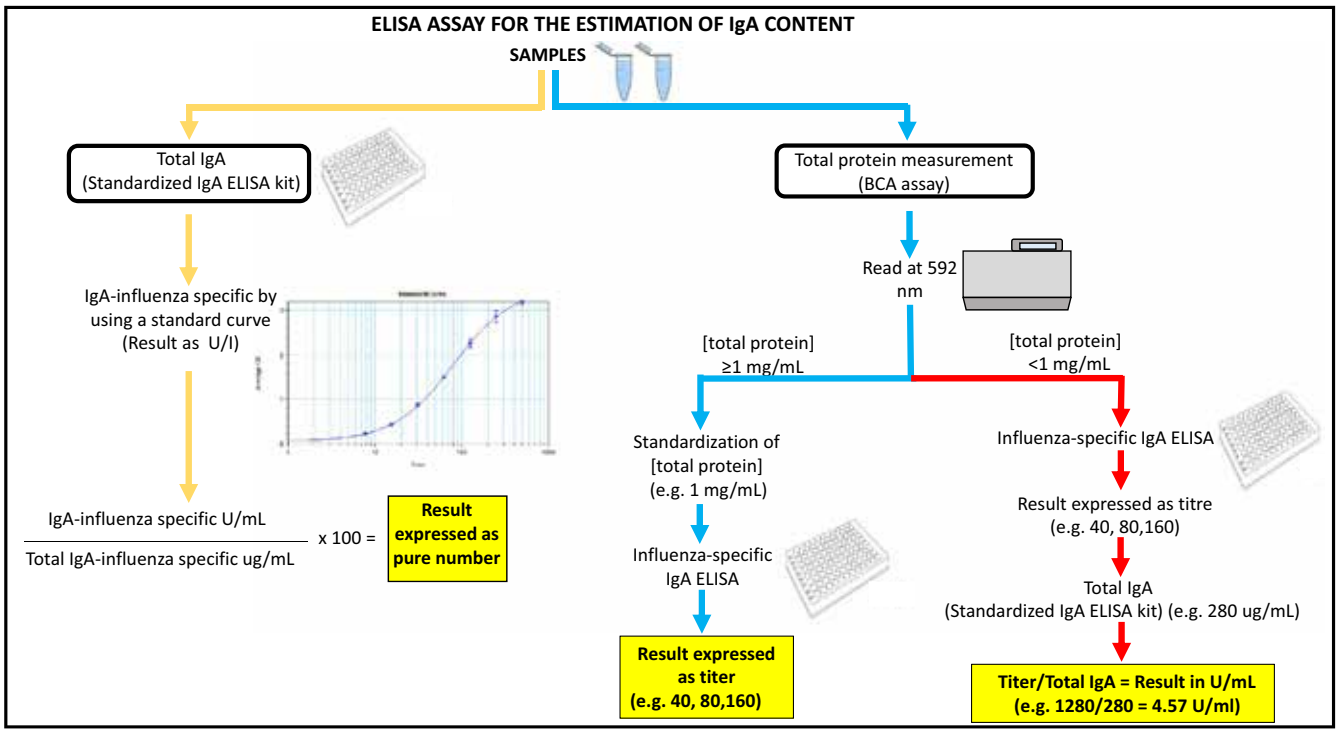

FIGURE 2 Principles of ELISA assays for the determination and standardization of the IgA content in test samples. Test samples include nasal washes, swabs, or saliva samples. Method 1: Standardization of antigen-specific IgA antibodies against total IgA (left side, yellow arrows). Method 2: Standardization of antigen-specific IgA antibodies against total protein (right side, blue and red arrows) Variation 1: high total protein content (>1 mg/mL; middle right side, blue arrows). Variation 2: low protein content (<1 mg/mL) or big differences of the total protein content of different test samples (rightmost side, red arrows)

immunosorbent assay (ELISA) is the most favorable one to measure the mucosal immune responses.

Enzyme-linked immunosorbent assay can accurately measure the concentrations of different classes of antibodies that are able to bind to influenza virions or purified HA proteins.[70] Many different protocols and standard kits are currently available on the market, but they are all based on the same principle. Generally, HA protein or whole influenza virus is pre-adsorbed to the wells of an ELISA microplate; different sample dilutions are then added, followed by the addition of the labeled secondary antibody, which is able to detect the immunoglobulins of interest. A colorimetric reaction is obtained upon the addition of a substrate. An important feature of the ELISA assay is that it can measure different classes of IgG, IgM, and IgA present both in serum and in mucosal samples.[71-73]

Currently, there are two main methods (Figure 2) of detecting influenza-specific sIgA responses in nasal wash/swab and saliva samples.[74,75] The two methods differ mainly in terms of the strategy adopted for the standardization of the samples to be analyzed, which precedes influenza-specific IgA detection. Standardization of the mucosal samples is an important step, since the mucus and protein concentration of nasal washes varies widely between individuals, depending on several factors, such as age, history or concurrence of nasal disease, and aspiration efficacy.[14] The first method described here is based on sample standardization according to the total content of IgA present in each sample by using a standardized IgA ELISA kit, whereas the second method is based on the quantification of total protein content through the bicinchoninic acid (BCA) assay (Figure 2).

4.1 | Influenza HA-Specific IgA respect to Total IgA

This method is used to normalize the influenza-specific IgA content of a sample through the total IgA content (Figure 2). The total IgA concentration in nasal wash/swab samples or saliva can easily be measured by using one of the many standardized ELISA kits available on the market. Concerning influenza-specific IgA detection, the procedure needs to be adapted due to the absence of a standardized human influenza-specific IgA reference. The ELISA procedure in principle has been described elsewhere.[73] In brief, ELISA plates are coated with an influenza antigen (preferably purified HA) and a capture antibody (anti-human IgA). Samples and standards are added to the plate and incubated for 1-2 hours at 37°C. The presence and concentration of influenza-specific IgA or total IgA is then determined by a color reaction applying an enzyme-labeled second antibody against human IgA and the respective substrate followed by a read-out in a conventional microtiter plate ELISA reader. The anti-influenza IgA concentration is being extrapolated from the standard regression curve derived by diluting a human total IgA reference standard of known starting concentration. As a consequence of this calculation, it is not possible to report the relative value of anti-influenza IgA as µg; instead, it should be expressed as Unit/mL (U/mL), where 1 U corresponds to 1 µg of human IgA detected. It is

important to run multiple samples collected from the same subject at different time points in the same ELISA plate.14 According to this method, the value of influenza-specific IgA normalized through the total IgA content will be expressed as *"(Influenza-Specific IgA (U/mL)/ Total IgA (μg/mL)) * 100."*.

4.2 | Influenza HA-Specific IgA and Total Protein

The basis of this method is the measurement of the total protein content in the samples (Figure 2). The determination of influenza HA-specific IgA with respect to total protein generally proves to be the best choice when a large number of samples have to be evaluated, since it is easy to use, sensitive, and rapid.[75] Depending on the total protein concentration obtained, two different methods of calculations can be adopted (Figure 2).

The first method will be applied if the total protein of the samples is higher than or equal to 1 mg/mL by standardization of nasal or saliva samples to a defined total protein content (Figure 2) which may vary according to the type of sample (nasal wash vs nasal swab vs saliva). The influenza-specific IgA antibody titer is then calculated as the reciprocal of the highest dilution that yields an OD signal greater than or equal to a predefined cutoff value. However, since completely negative human nasal samples are usually not available, the exact calculation of a cutoff may not be optimal and require alternative approaches in the future.

One approach may be to use the "limit of blank" according to the following formula: *"Average of background signals (OD_{Blank}) plus 2 standard deviations."*[72] In this case, the cutoff value will be calculated without the need for a specific human sample; only ELISA reagents will be added to the coated plate together with the influenza antigen, and the background signal will be used to calculate the cutoff. An alternative possibility is the calculation of the cutoff value as the reciprocal of the highest dilution that shows an absorbance value >0.2 of the OD value after subtraction of the background as previously described.[73]

In the case of low total protein concentrations of the samples in general or of big differences of the total protein content of different test samples, an alternative approach, combining the two approaches described above, can be used which is based on the estimation of IgA content by using the ratio between the titer and the total IgA content (Figure 2).

5 | NEUTRALIZING (NT) ANTIBODIES IN NASAL WASH/SWAB AND FUTURE ASSAYS

Some recent studies[74,76] have assessed neutralization (NT) antibody levels in standardized nasal wash/swab samples after intranasal immunization since NT antibodies are generally considered more specific than hemagglutination inhibition (HI) antibody titers in children vaccinated with LAIVs. However, it has been shown in a previous pediatric study with LAIV that influenza virus–specific salivary IgA levels correlated with serum HI responses,[76] although it is also discussed that the measured HI titers may underestimate the protective potential of LAIVs.[60,77] NT antibodies in serum samples are usually assessed by means of the microneutralization (MN), either CPE (cytopathic effect)-based[74] or ELISA-based,[37] or the plaque-reduction neutralization (PRNT) assay. In the present review, we focused on the CPE-based MN assay, since this is the preferred method because of its simplicity of execution, its ability to evaluate large numbers of samples, and the standardization of the quantity of virus used in the assay.[78] Along with the ELISA sIgA assay, the MN assay constitutes a valid approach to evaluate the immunogenicity of LAIVs, IIVs, or recombinant influenza vaccines (eg rHA) in inducing selective anti-influenza antibodies with influenza virus–neutralizing potential.

Beside classical ELISA-based and NT assays specific anti-HA influenza antibodies, there are newer assays with increased precision and sensitivity, such as the XMAP (x = analyte MAP = Multi-analite profiling) technology adapted for Luminex-based IgA assays.[79,80] The XMAP technology is a serological method that can be applied to measure multiple proteins or antibodies in a single-well reaction with high accuracy and reproducibility. The Luminex XMAP technology is based on the combination of different well-established techniques such as flow cytometry, carboxylated microspheres, laser, and traditional chemistry. Briefly, specific nano-magnetic beads can be coated with different purified proteins arising from the same pathogen or from different ones and then incubated with serum samples. Using specific biotinylated secondary antibody, the presence of antigen-specific antibodies in sera can be easily measured and quantified by a dedicated detection system (Luminex). Wang et al[81] applied this novel method for the simultaneous detection of antibodies against the Newcastle disease and avian influenza virus and have shown that the Luminex XMAP-based assay has been up to 1024 times more sensitive for avian influenza virus antibody detection compared to the conventional ELISA assay. The minimal volume of sample required, the cost reduction for multiple detection in comparison with the classical methods, and the possibility to perform a rapid multiplexing in a single reaction are additional advantages of this new technology. However, these new generations of serological assays are not standardized and require further studies for the generation of validated and reproducible results. Currently, only the ELISA is a reliable and valuable approach to determine sIgA in various biological samples.

6 | CONCLUSIONS

Influenza vaccines elicit protective immunity before a new influenza virus variant is able to spread; they therefore constitute a primary protection tool. Although the main protective effectors against influenza virus infection are CTLs, IgG, and IgA located in the respiratory mucosa, most of the vaccines currently available are inactivated vaccines that are administered via parenteral injection, and which mostly promote serum IgG rather than mucosal IgA (rev. in[82-84]). The importance of intranasally applied LAIVs is their ability to reproduce a natural infection without causing disease or virus transmission. They mimic the natural encounter with the antigen by activating the innate immune system and promoting antibody and T cell–mediated

immune responses. This type of vaccine can induce a broader immune response in children than intramuscular vaccines.[31,85,86] Furthermore, mucosal vaccines can elicit cross-reactive antibodies in humans. However, the development of cross-protective T lymphocytes has been observed in animal models, but this has not yet confirmed in humans.[85,86]

An additional advantage of LAIVs is their consumer-friendly needle-free intranasal application which represents a minimal invasive delivery method, and it is expected with higher production capacities and a more widely distribution. For these reasons, its expanded use could increase the influenza vaccination coverage globally. Furthermore, it may represent a favorable approach for mass immunizations, especially in younger children since its application is not associated with pain.[87]

Although LAIVs have been on the global market for many years, no established correlates of protection for them are yet available.[77] Moreover, previously reported discrepancies of efficacy data from Europe and the US further complicate the understanding of the immune response elicited by LAIV.[77] Despite these complications, great efforts have been made in the recent years to develop novel intranasally administered vaccines to promote influenza virus-specific sIgA,[30,88] which, as has been widely reported, provide broader protection than serum IgG. A robust mucosal response is fundamental in order to protect both the single individual and the entire population by preventing transmission of the virus to susceptible subjects.[89] Notably, the use of the ELISA assay for IgA detection could play a major role in the evaluation of vaccine efficacy or effectiveness in the field, as currently influenza vaccine efficacy is traditionally assessed by means of serological assays that detect influenza-specific serum antibodies induced by the vaccine itself. However, these assays cannot be properly applied to intranasal vaccines, which mainly induce local immune responses (rev. in[90]).

In conclusion, the measurement of sIgA in mucosal secretions for the evaluation of influenza vaccine efficacy or effectiveness and, in addition, also of the effectiveness of vaccines against other respiratory virus infections of the respiratory mucosae, is arousing great interest and may constitute a valuable asset.

CONFLICT OF INTEREST

There are no conflicts of interest in the conduction of this study.

AUTHOR CONTRIBUTIONS

EG and A.M involved in writing, reviewing, and editing processes and prepared the images; O. K., CT, and I.M involved in reviewing and editing processes; and EM involved in supervision and review process.

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