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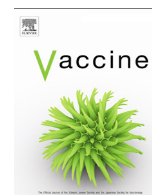

VaxArray assessment of influenza split vaccine potency and stability

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ABSTRACT

Vaccine manufacturers require more rapid and accurate tools to characterize the potency and stability of their products. Currently, the gold standard for influenza vaccine potency is the single radial immunodiffusion (SRD) assay, which has inherent disadvantages. The primary objective of this study was to investigate the ability of the VaxArray Influenza (VXI) seasonal hemagglutinin (sHA) potency assay to accurately quantify potency and stability in finished vaccines as well as to quantify hemagglutinin protein (HA) within crude in-process samples. Monobulk intermediates and mono- and multivalent vaccines were tested using VXI. Quantification of HA in crude samples was evaluated by spiking known concentrations of HA into allantoic fluid. VXI generated SRD equivalent potency measurements with high accuracy (within ±10%) and precision (CV 10 ± 4%) for antigen components of monobulk intermediates and multivalent split vaccines. For these vaccines and vaccine intermediates, the VXI linear dynamic range was ~0.01–0.6 µg/mL, which is 12× greater than the linear range of SRD. The measured sample limit of detection (LOD) for VXI varied from 0.005 to 0.01 µg/mL for the different subtypes, which in general is ≥600× lower than the LOD for SRD. VXI was able to quantify HA in crude samples where HA only accounts for 0.02% of the total protein content. Stability indication was investigated by tracking measured potency as a function of time at elevated temperature by both SRD and VXI. After 20 h at 56 °C, the ratio of VXI to SRD measured potency in a quadrivalent vaccine was 76%, 125%, 60%, and 98% for H1/California, H3/Switzerland, B/Phuket and B/Brisbane, respectively. Based on the study results, it is concluded that VXI is a rapid, multiplexed immunoassay that can be used to accurately determine flu vaccine potency and stability in finished product and in crude samples from upstream processes.

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1. Introduction

Seasonal and pandemic influenza infections pose significant public health threats. The rapid development of vaccines serves as a foundation for the prevention of seasonal and pandemic influenza outbreaks. The trivalent and quadrivalent split influenza vaccines tested in this study were developed by GlaxoSmithKline (GSK) for active immunization for the prevention of disease caused by influenza A subtype viruses and type B viruses contained in the vaccine. According to guidelines provided by the World Health Organization, vaccine producers must determine the potency at the time of release and throughout the approved shelf life of the product [1]. Potency assays measure the concentration of the influenza surface protein hemagglutinin, which has been established to be the dominant target of protective antibodies following vaccination or infection [2,3]. The single radial immunodiffusion assay (SRD aka SRID) measures the immunological reaction between antisera and test antigen and is the only current internationally recognized method for establishing and tracking influenza vaccine potency and stability [4]. Developed in 1978, SRD is a labor intensive assay that relies on seasonal reference reagents that result from a complex interaction between surveillance laboratories, vaccine producers, and regulatory agencies. New reference reagents must be developed when a strain change is required for the seasonal vaccine and this process can take up to four months, thereby complicating the vaccine development. Furthermore, it has been acknowledged that potency determined by SRD does not provide "*an exact correlate between vaccine potency and clinical outcome*" [5,6]. For these reasons and others [7,8], there is an extensive effort to develop and test alternative influenza vaccine potency assays.

The technologies currently being examined as replacements for SRD include HPLC [9–11], surface plasmon resonance (SPR) [12,13], mass spectrometry [14–17], and several different immunoassays [18–22]. There are limitations associated with each technology. For example, immunoassays require well-characterized antibody reagents. HPLC [23] and mass spectrometry methods [15] require

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complex sample preparation and non-biologically relevant sample conditions. While offering improved sensitivity to SRD, adsorption methods based on SPR detection (e.g., Biacore) [12,13], are generally considered too expensive [6]. Additionally most of the published alternative potency assays, like SRD, are limited to analysis of a single antigen component during measurement, which can be tedious, time-consuming, and materials intensive.

Despite reliance on specific antibodies, immunoassays offer distinct advantages for quantifying structurally intact proteins and therefore tracking protein stability in addition to providing a direct measure of HA concentration [12,14,18–20]. The stability indication capability of a potency assay is crucial because licensure of influenza vaccines requires potency determination as well as evaluation of stability in both accelerated and real-time testing [24]. Due to the time-sensitive nature of producing seasonal vaccines on a yearly basis, forced degradation (accelerated) studies are considered to be critical tools for quickly assessing stability [25].

The goal of this work was to evaluate the VaxArray platform as a rapid influenza vaccine potency assay for use with seasonal vaccines. VXI is a simple multiplexed sandwich immunoassay that utilizes a glass substrate printed with broadly reactive yet subtype specific antibodies for A/H1, A/H3, B/Yamagata-like, and B/Victoria-like strains in a microarray format [20]. A "universal" polyclonal label antibody is used to quantify all components of monovalent and multivalent HA mixtures. In a previous study, VaxArray (formerly known as Titer on a Chip) generated nearly equivalent potency determination relative to SRD for *recombinant HA* produced in a baculovirus expression system [20]. This study expands on the previous work to include the suitability of VXI for potency determination and stability indication of mono- and multivalent *split virus vaccine*s produced in eggs. The vaccines used in this study were developed by vaccine manufacturer GSK for the 2015–2016 flu season.

2. Materials and methods

2.1. Sample receipt and handling

Eight monovalent intermediate bulks, two monovalent vaccines, and four multivalent vaccines were provided by GSK, along with GSK in-house standards and NIBSC reference standards (see Tables 1 and 2). The strain-specific GSK-in-house standards were obtained from purified split virus fractions originating from commercial vaccine manufacturing (proprietary technology) and were calibrated for their HA content using SRD, SDS-PAGE and HA-HPLC (see below). Upon receipt, all samples and GSK standards were stored at 4 °C. Lyophilized standards from NIBSC and CBER were stored at −20 °C and then at 4 °C once reconstituted with water as per use specifications.

2.2. HA quantification using VaxArray seasonal influenza potency assay

The assay and array layout for VXI are illustrated in Fig. 1A-B. Briefly, HA proteins are captured by sub-type specific monoclonal "capture" antibodies and detected by a "universal" polyclonal antibody conjugated with a "Cy3" equivalent fluorophore (excitation at 532 nm and emission at 570 nm). The array can be used for simultaneous analysis of HA proteins from A/H1, A/H3, B/Yamagata-like and B/Victoria-like influenza viruses.

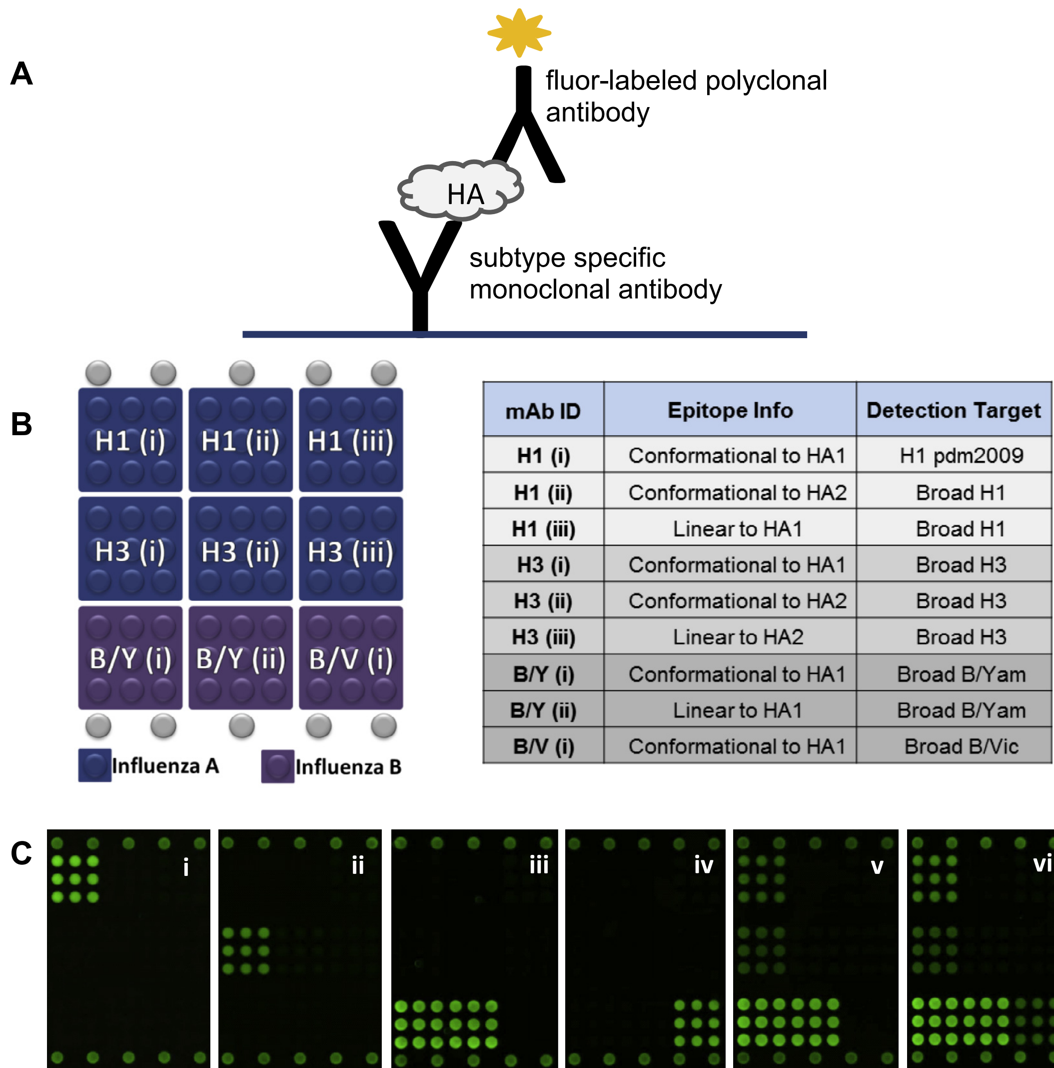
VXI reagents kits (#6500, InDevR) contain two microarray slides (75 × 25 mm), each printed with 16 arrays per slide, Positive Control Label, Protein Blocking Buffer (PBB), and two Wash Buffers. Prior to use, VXI slides were removed from the refrigerator and equilibrated at room temperature for 30 min in their foil pouch. For quantification in this study, eight arrays were used for an 8-point calibration curve and up to 24 arrays were used for

Table 1
Standards used in this study.

Reference antigen	Lot # or ID	Source	Concentration by SRD (μg/mL)	Concentration by SDS-Page (μg/mL)
A/Christchurch/15/2010 (H1N1) (NIB-74)	10/258 (29 μg/mL)	NIBSC	NA	NA
A/Switzerland/9715293/2013 (NIB-88)	14/254 (55 μg/mL)	NIBSC	NA	NA
B/Phuket/3073/2013	14/252 (32 μg/mL)	NIBSC	NA	NA
B/Brisbane/60/08	13/234 (42 μg/mL)	NIBSC	NA	NA
A/Shanghai/02/2013	78 (60 μg/mL)	CBER	NA	NA
H1N1 Standard A/Christ/16/10 NIB-74XP	3-A/Christ (Standard)	GSK In-house split virus (MB)	180	164
H3N2 Standard A/Switz/9715293/2013 NIB-88	4-A/Switz (Standard)	GSK In-house split virus (MB)	217	183
B/Yamagata-like Standard B/Phuket/3073/2013	10-B/Phuk (Standard)	GSK In-house split virus (MB)	250	341
B/Victoria-like Standard B/Brisbane/60/2008	9-B/Bris (Standard)	GSK In-house split virus (MB)	176	347
H7N9 Standard A/Shanghai/02/2013	14-H7N9 (Standard)	GSK In-house split virus (MB)	121 (10/2013); 88 (01/2014)	120

Table 2
Samples analyzed in this study.

Samples received for analysis by VaxArray		
Sample ID	Type	Virus Strain
1-A/Christ	Monovalent Intermediate Bulk	H1N1 A/Christ/16/10 NIB-74xP
2-A/Christ	Monovalent Intermediate Bulk	H1N1 A/Christ/16/10 NIB-74xP
5-A/Switz	Monovalent Intermediate Bulk	H3N2 SWITZ/9715293/2013 NIB-88
6-A/Switz	Monovalent Intermediate Bulk	H3N2 SWITZ/9715293/2013 NIB-88
7-B/Bris	Monovalent Intermediate Bulk	B/vic-like Brisbane/60/2008
8-B/Bris	Monovalent Intermediate Bulk	B/vic-like Brisbane/60/2008
11-B/Phuk	Monovalent Intermediate Bulk	B/Yam-like Phuket/3073/2013
12-B/Phuk	Monovalent Intermediate Bulk	B/Yam-like Phuket/3073/2013
13-H7N9	Monovalent Vaccine	A/Shanghai/02/2013
15-TIV	Trivalent Vaccine	H1N1, H3N2 and B/Yam-like Phuket/3073/2013
17-TIV	Trivalent Vaccine	H1N1, H3N2 and B/Yam-like Phuket/3073/2013
16-QIV	Quadrivalent Vaccine	H1N1, H3N2, B/Yam-like Phuket/3073/2013 and B/vic-like Brisbane/60/2008
18-QIV	Quadrivalent Vaccine	H1N1, H3N2, B/Yam-like Phuket/3073/2013 and B/vic-like Brisbane/60/2008
19-Pan	Monovalent Vaccine	A/Shanghai/02/2013



mAb ID	Epitope Info	Detection Target
H1 (i)	Conformational to HA1	H1 pdm2009
H1 (ii)	Conformational to HA2	Broad H1
H1 (iii)	Linear to HA1	Broad H1
H3 (i)	Conformational to HA1	Broad H3
H3 (ii)	Conformational to HA2	Broad H3
H3 (iii)	Linear to HA2	Broad H3
B/Y (i)	Conformational to HA1	Broad B/Yam
B/Y (ii)	Linear to HA1	Broad B/Yam
B/V (i)	Conformational to HA1	Broad B/Vic

Fig. 1. VaxArray VXI v1.2. (A) Illustration of the immunoassay. (B) Schematic of the VXI v1.2 array layout of subtype specific antibodies to A/H1, A/H3 subtypes as well as B/Yamagata and B/Victoria lineages. The array contains 9 replicate spots (~200 μm in diameter) of each monoclonal antibody. Table provides epitope information and reactivity for each monoclonal antibody. (C) Representative fluorescence images for the following vaccines on the VXI microarray: (i.) H1/Christchurch monovalent, (ii.) H3/Switzerland monovalent, (iii.) B/Phuket monovalent (Yamagata lineage), (iv.) B/Brisbane monovalent (Victoria lineage), (v.) Trivalent vaccine composed of H1, H3, and B/Yam-like, and (vi.) Quadrivalent vaccine composed of H1, H3, B/Yam-like, and B/vic-like. The brightness of the green spots is an indication of signal intensity.

samples. The samples were processed by the method described in the VaxArray Operation Manual (R003). In summary, standards and samples were diluted with PBS and treated with 1% Zwittergent 3–14 for 30 min. Each standard was then serially diluted with Protein Blocking Buffer/1% Zwittergent (PBBZ) to make 8 calibration standards. PBBZ was also added to each sample to yield the final dilutions for analysis. After placing the slides in a humidity chamber, 50 μL of each standard was applied to wells on the left side of the slide, and 50 μL of each sample was added to the remaining wells in triplicate and incubated in a dark humidity chamber for one hour. Samples were quantified against both GSK in-house standards and SRD reference reagents. The antigens were removed and 50 μL of label, consisting of a mix of the Positive Control Label and Polyclonal A/B Label (#5514, InDevR) in PBB was applied and incubated for 30 min. Label was removed with an 8-channel pipette and slides were sequentially washed with Wash Buffer 1, Wash Buffer 2, and 70% ethanol using a wash bin. The ethanol was removed using an air source and the back of each slide was washed with a tissue wetted with 70% ethanol, dried with a clean tissue wipe, and placed in a drying box for ~10 min. Imaging was conducted on a Vidia Microarray Imaging System (InDevR), which has LED excitation centered at 530 nm and fluorescence emission collection at 570 nm. Image collection times ranged from 400 ms to 1000 ms per array. Data was automatically processed using the VaxArray Processing Workbook v1.2 described by Kuck et al. [20]. The linear ranges were automatically calculated, plotted and the HA concentration measured in triplicate for each sample was automatically averaged. A concatenated image of all 16 arrays for each slide was also created as a record.

2.3. Forced degradation studies

For four monovalent intermediate bulks and one quadrivalent vaccine, 100 μL aliquots were added to 1.5 mL amber glass vials, sealed with a crimp top, and weighed. Samples were heated in a water bath for 20 h (T20), 8 h (T8), 5 h (T5), and 1 h (T1) while a control was retained at 4 °C (T0). The water temperature was continuously monitored and was 55–56 °C during the entire degradation time period. After degradation, the vials were briefly cooled on ice and then stored at 4 °C until analysis later that day. Each vial

was re-weighed before analysis to check for inadequate sealing and possible evaporation during degradation. All weights showed <1% difference after degradation. For the quadrivalent vaccine, the non-degraded sample was used as the VaxArray calibration standard.

2.4. In-process crude sample analysis

A quadrivalent vaccine (sample #16) was diluted to 5 μg/mL in both PBS and allantoic fluid (Virapur Uninfected Allantoic fluid, Lot# A1318A) and lysed according to the VXI operation manual. Both samples were diluted to 0.25 μg/mL in PBBZ. The two samples and a blank (allantoic fluid) were quantified in triplicate against a quadrivalent calibration curve. The calibration curve was generated by treating GSK Sample #16 in PBS with 1% Zwittergent before diluting to 0.60 μg/mL in PBBZ and serially diluting in PBBZ to yield calibration standards.

2.5. Single radial immunodiffusion analysis (SRD)

SRD was performed as specified in the protocol "Potency Determination of Inactivated Influenza Virus Vaccines by the Single Radial Immunodiffusion Method" (CBER, Document ID 000333, Release Date 9/23/14) with minor modifications to the protocol. Specifically, the samples and GSK in-house standards were determined with three independent replicates. The reference antigens were diluted to ~30 μg/ml HA and subsequently serially diluted to 100%, 75%, 50% and 25% to obtain ~30, 22.5, 15, and 7.5 μg/ml standards. The samples were diluted in the same manner. For SRD of B-strains in quadrivalent vaccine samples, SRD was modified using both B strain reference antigens pooled 1:1 to yield a final HA concentration of 30 μg/ml for each B-strain in the pooled standard. Samples and reference antigens were incubated with 10% Zwittergent solution for 30 min.

The 1% Agarose gels were prepared using agarose melted at 56 °C ± 1 °C and poured after addition of strain-specific antisera onto pre-coated glass plates (25 mL on approximately 100 × 150 mm). A total of 10 μL of each replicate sample, in-house standard and reference antigen was pipetted threefold into punched 3 mm wells in a randomized loading scheme and incubated for at least 18 h at 20–25 °C. The plates were evaluated for the precipitation rings using an automatic reading system (Axiovision, Zeiss). The HA concentrations were calculated using a slope ratio-method [26].

2.6. SDS-PAGE determination of HA concentration

The Mini-PROTEAN® 3 Cell and ready-to-use gels (TGX®) (Bio-Rad Laboratories) were used and the method was performed as described previously [27]. The band pattern in the Coomassie®-stained gels was analyzed using an automated gel reader (Power Scan 900, Biosteps). The HA content for related protein bands was calculated from the respective densitometric peak area in relation to the total protein content determined by chemiluminescence nitrogen assay (Multi-NC, Analytic Jena).

2.7. HPLC determination of HA concentration

The HPLC method targets HA subunit 1 (HA_{su1}) obtained by a DL-dithiothreitol (DTT) reduction step. The assay requires the generation of a calibration curve with a monovalent strain of the respective NIBSC reference antigen. For the assay performance samples and standards were added to a mixture of SDS and DTT and appropriately diluted with purified water. The standards were diluted to concentrations of 50, 40, 30, 15, and 5 μg/mL HA. Samples and standards were boiled for 15 min. The resulting solutions were subjected to reverse-phase HPLC analysis using a Thermo Fisher HPLC system. The 15 min separation was conducted in an Agilent Poroshell 300 SB C3 column maintained at 60 °C. An acetonitrile/water linear gradient from 26% to 41% acetonitrile was used at a flowrate of 0.55 mL/min with starting concentration of trifluoroacetic acid (TFA) of 0.1% (w/v) and an ending concentration of 0.0925% (w/v). The chromatograms were acquired at 212 nm using a photo diode array (PDA) detector. The vials were kept at room temperature in the autosampler. The HA concentration of the samples was calculated by comparing the peak areas of the HA-related peak against the appropriate calibration curve.

3. Results and discussion

3.1. VXI qualitative response to seasonal mono- and multivalent split virus vaccines

Tables 1 and 2 summarize the standards and samples analyzed in this study. A schematic illustrating the assay is present in Fig. 1A. The location and specificity of each antibody on the array is presented in Fig. 1B with a table that describes antibody epitope information. In the VXI assay, HA proteins are captured by subtype specific monoclonal "capture" antibodies and detected by a universal polyclonal antibody conjugated with a proprietary fluorescent label (Fig. 1B). Representative fluorescent images for monobulk as well as trivalent and quadrivalent vaccines are shown in Fig. 1C. Since the VaxArray platform is ideally suited for multiplex analysis, it is important to quantify any cross-reactivity on the array. To assess cross-reactivity, the signal intensity observed on each capture mAb was quantified for each monovalent antigen using a concentration at the top end of the linear dynamic range. Panels i-iv in Fig. 1C show images of monovalent samples at 0.5 μg/mL; analysis of the signal intensity on all non-target antibodies yielded values at background levels (i.e., below the detection limit). No cross-reactivity was observed for any of the monovalent samples at concentrations within the linear dynamic range; thus, the capture antibodies react in a subtype-specific manner and can be used to quantify individual subtypes. With this subtype specificity the microarray platform is applicable to analysis of each antigen within multivalent mixtures in a single multiplexed experiment, a capability that no other potency assay has demonstrated.

3.2. The calibration standard dilemma

Despite concerns over the reliability of SRD potency measurements to serve as a reliable predictor of vaccine efficacy [5,6], it is generally agreed that the accuracy of an alternate potency assay must be judged relative to SRD. However, the reference antigens developed for SRD do not necessarily adequately represent the composition of vaccines. This is highlighted by the requirement of having different reference antigens for vaccines produced in eggs and cell culture [28]. The issue is even further emphasized by the potency measurement challenges faced by emerging vaccine technologies, such as those based on recombinant proteins or virus-like particles [8].

For VXI, the difference in response for the traditional reference antigens (e.g., CBER or NIBSC) relative to an internal reference antigen (formulated to be similar in composition to a vaccine) is demonstrated in the serial dilution curves shown in Fig. 2 with error presented as the standard deviation across 9 antibody spots per subtype. The slope of the calibration curve of the split virus vaccine-like internal reference antigen is nearly two times that of the NIBSC standard. One possible contributing factor is potential differences in protein quaternary structure(s) for the split virus

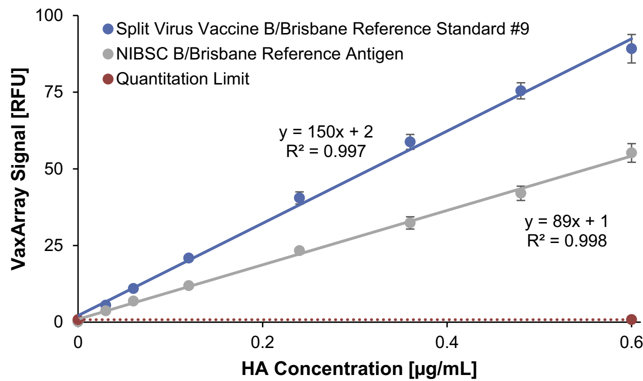


Fig. 2. Calibration differences between antigen types for B/Brisbane. Serial dilution plots showing response differences for reference standards (NIBSC Lot 13/234) in green (whole viruses, split just prior to analysis) versus internal standards (#9) in blue (split virus with additional purification steps). The red dotted line represents the measured quantification limit of the assay. Errors bars represent the standard deviation across 9 antibody replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

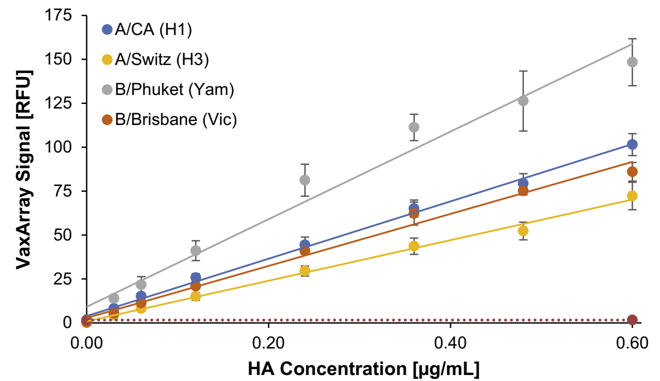


Fig. 3. Representative calibration curve for each reference strain within quadrivalent split virus vaccine sample #16. The solid lines represent linear regressions to all data points in each curve. However, it is important to note that the VXI assay relies on automatic analysis that determines linear response ranges within the calibration curve via 4-point linear regression analysis as described previously [20]. Errors bars represent the standard deviation across 9 antibody replicates.

vaccine-like internal reference antigen versus the whole virus traditional reference standard [29]. For example, aggregates of the HA trimer, such as rosettes, could affect epitope availability and thus concentration measurements. However, based on preliminary studies, a working hypothesis is that the primary cause is associated with differences in the degree of chemical modification of the reference antigens relative to the vaccines, which results in reduced binding avidity of the reference antigens in the VXI assay. Work is ongoing to better understand and address the calibration standard issue; however, all of the data presented in this paper are based on quantification against internal standards developed and pre-characterized by GSK using SRD, HPLC, and SDS-PAGE. Representative calibration curves for each subtype are shown in Fig. 3 with error presented as the standard deviation across 9 antibody spots per subtype.

3.3. Quantification of seasonal mono- and multivalent split virus vaccines using VXI

3.3.1. Accuracy

To assess the accuracy of VXI, a total of 12 samples were run in triplicate on three different days and compared to SRD values that had been previously obtained by GSK. The accuracy of VXI was assessed for each subtype within monobulk intermediates, monovalent vaccines, and multivalent vaccines by generating "percent of SRD" values (i.e., (VXI value/SRD value) $*$ 100). Percent of SRD values for each antigen in four monovalent samples, two trivalent vaccines, and two quadrivalent vaccines are reported in Fig. 4. The average strain-specific relative potency measured by VXI with standard error of the mean for all samples (based on 189 total measurements) is 96 ± 2% for H1, 90 ± 3% for H3, 109 ± 1% for B/Phuket, and 98 ± 3% for B/Brisbane. For one of the two monovalent sample sets (Monovalent 1, Fig. 4B), good agreement between SRD and VXI was observed (95% for H1, 99% for H3, 107% for B/Phuket, 106% for B/Brisbane). However, a discrepancy was consistently measured for the second monovalent sample set (Monovalent 2, Fig. 4B) for H1, H3, and B/Brisbane subtypes (65% for H1, 55% for H3, 75% for B/Brisbane). One possible explanation is use of a non-homologous reference antigen, which is being further investigated. For the multivalent vaccines, as shown in Fig. 4, nearly equivalent results were obtained by VXI and SRD for all HA subtypes. For multivalent vaccines, the average VXI measured potency is nearly identical to SRD with %SRD values of 104 ± 2% for H1, 96 ± 2% for H3, 109 ± 2% for B/Phuket, and 105 ± 3% for B/Brisbane.

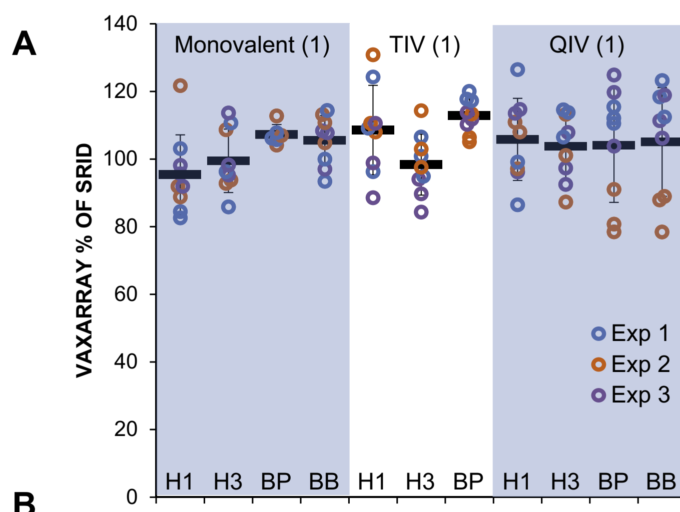
3.3.2. Precision

For all samples tested in this study, the precision was determined for daily triplicate measurements, day-to-day averages, and pooled averages (data not shown). Triplicate measurements for all monovalent and quadrivalent samples yielded an average relative error of 8 ± 5%, and the average day-to-day relative error for all samples was 6 ± 5%. The pooled precision for all antigens over all VXI measurements made in this study resulted in an average relative error of 10 ± 4%. Given that relative error for SRD experiments has been reported to be ~10% [30], VXI performs similarly to SRD in terms of precision.

3.3.3. Limit of detection and linear dynamic range

The limit of detection and linear dynamic range for each subtype was quantified using the GSK in-house standards. The instrumental limit of detection ($LOD_{inst.}$) was defined as the average signal from a blank sample plus three times the measured standard deviation. Since the VXI assay involves a minimum overall sample dilution of ~3 (i.e., dilution with zwittergent and 1:1 dilution into PBB), the $LOD_{inst.}$ was multiplied by the dilution factor to generate a typical *sample* LOD ($LOD_{samp.}$) value. Using this approach, $LOD_{samp.}$ values were determined for each subtype within the mono- and multivalent vaccines in this study. For monovalent antigens, the respective LOD_{samp} values are 0.0046 µg/mL for H1, 0.0100 µg/mL for H3, 0.0072 µg/mL for B/Phuket, and 0.0078 µg/mL for B/Brisbane. The LOD_{samp} of VXI is ≥600 times lower than the LOD of SRD (~6 µg/mL). The VXI assay is sufficiently sensitive that samples were diluted by factors ranging from ~200–1200 before analysis, dramatically reducing the amount of analyte required for VXI compared to SRD. While such large dilution factors can in some case reduce overall assay precision, this error can be mitigated when high precision is needed by using measured mass rather than relying on pipetted volumes.

Representative calibration curves for each component of a quadrivalent split virus vaccine are shown in Fig. 3. As tested simultaneously in the multiplexed format, all four components of a quadrivalent vaccine exhibited a linear dynamic range of ~0.01–0.6 µg/mL. The 60-fold quantification range is 12 times better than the quantification range for SRD (typically 6–30 µg/mL). Due to the multiplexed nature of the assay, only a single quadrivalent standard was needed, as opposed to the four separate SRD analyses that would be required to quantify each of the HA subtypes. Adding to this point, because the assay has better sensitivity, 30 times less standard material is needed for a monovalent stan-



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SAMPLE	H1	H3	BP	BB	H7
Monovalent 1	95 ± 4	99 ± 3	107 ± 1	106 ± 2	100 ± 6
Monovalent 2	65 ± 3	55 ± 3	108 ± 4	75 ± 2	81 ± 2
TIV 1	109 ± 4	98 ± 3	113 ± 2		
TIV 2	97 ± 3	100 ± 3	107 ± 2		
QIV 1	106 ± 4	104 ± 3	104 ± 6	105 ± 5	
QIV 2	103 ± 2	81 ± 2	111 ± 3	105 ± 4	
AVERAGE:	96	90	109	98	91
SEM:	2	3	1	3	5
N (total samples):	53	53	47	36	11

Fig. 4. Summary of VaxArray results relative to SRD determined potency. (A) Univariate scatterplot representation of VXI potency values divided by SRD potency values (i.e., % of SRD) for monovalent samples 1, 5, 11 and 7, trivalent sample 15 and quadrivalent sample 16. Abbreviations are as follows: H1 for A/California H1N1, H3 for A/Switzerland H3N2, BP for a B/Phuket, BB for B/Brisbane, TIV for trivalent vaccine and QIV for quadrivalent vaccine. The assay was run on three different days and each data point is colored in accordance with the day it was run. Error bars represent the standard deviation for data from all three days combined. (B) Table representation of % SRD values for each subtype within monovalent, trivalent, and quadrivalent vaccines. Error is represented by the standard error of the mean (SEM).

dard curve than for SRD. It is also important to note that VXI requires no reference anti-sera. Currently, regulatory agencies must produce large quantities of both reference antigen and reference antisera for use in SRD.

3.3.4. VXI quantification of crude in-process samples

Another drawback to SRD is that the assay does not work well with crude samples. It was demonstrated previously that VXI can be used for the quantification of recombinant HA in crude extracts from cell culture where the antigen concentration is low and the host protein contaminants are high [20]. To determine whether or not a crude protein-rich matrix like allantoic fluid would interfere with the VXI assay, minimally diluted allantoic fluid was analyzed on VXI (Fig. 5A, panel i). Analysis of the signal intensity of all antibodies on the array yielded values at background levels suggesting no interference by allantoic fluid. To evaluate the ability of VXI to quantify HA during the early stages of an egg-based manufacturing process, a quadrivalent split virus vaccine was diluted to 5 μg/mL HA in allantoic fluid, with a total allantoic protein concentration of 25 mg/mL, and in PBS, as a negative control, and lysed in 1% Zwittergent. After lysis, the mixtures were diluted to 0.25 μg/mL HA in PBBZ and analyzed by VXI. In this test case, HA only accounted for 0.02% of the total protein in the sample diluted in allantoic fluid. The corresponding VXI images are shown in Fig. 5, panels ii-iii. Average measured HA concentrations from triplicate measurements of both samples were 0.26 ± 0.01 μg/mL and 0.27 ± 0.03 μg/mL for each of the four subtypes diluted in allantoic fluid and PBS, respectfully (Fig. 5B), with error reported as standard deviation. These results confirm that VXI can be used in the upstream steps of vaccine manufacturing, providing manufacturers with a simple way to monitor HA production and yield throughout the inoculation, amplification, and purification steps of vaccine production.

3.3.5. VXI stability indication capabilities

To investigate the utility of VXI for tracking HA stability, a forced thermal degradation experiment using monobulk intermediates and multivalent vaccines was performed. Briefly, samples were analyzed before (T0) and after 20 h at 56 °C (T20) and%T0 values were calculated as (as $\frac{T20}{T0} \times 100$). The following three potency assays were compared for their stability indication capabilities: VXI, SRD, and HPLC. VXI and SRD were performed in triplicate for each sample, while HPLC was performed in duplicate for each sample. Results demonstrating the stability indicating properties of the three platforms, in terms of%T0, are summarized in Fig. 6. As shown in Fig. 6A, after a 20-h incubation at 56 °C the VXI-measured HA concentrations within a quadrivalent vaccine decreased to 18% of the non-degraded HA concentration for H1, to 49% for H3, to 23% for B/Phuket, and to 32% for B/Brisbane. Interestingly, monobulk samples of the same antigens exhibited much less degradation when exposed to the same thermal stress; as shown in Fig. 6B the%T0 values for VXI were 41% (vs. 18% in QIV) for H1, 109% (vs. 49%) for H3, 47% (vs. 23%) for B/Phuket, and 79% (vs. 32%) for B/Brisbane. Reasonable explanations for the observed differences in measured stability for multivalent vaccines versus monobulks, with monobulks being more stable, include

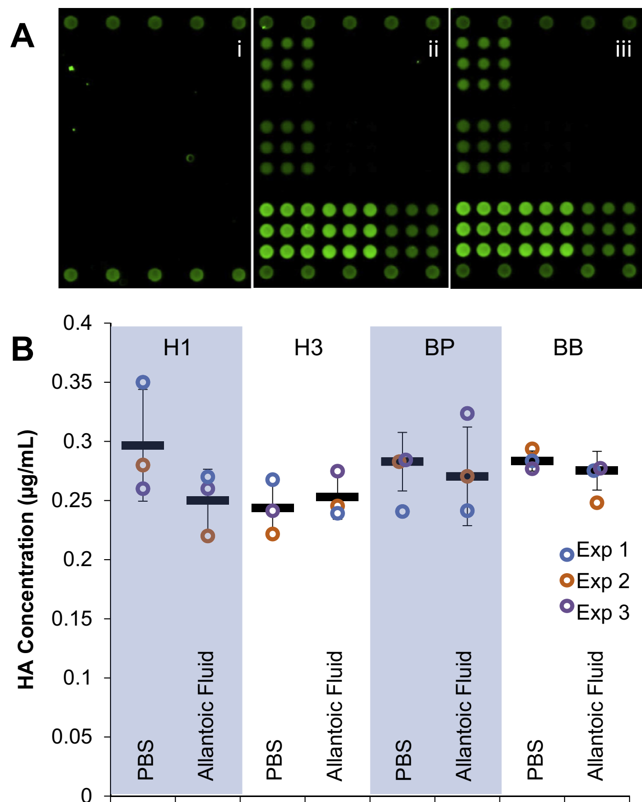

Fig. 5. VXI analysis of crude samples. (A) VXI fluorescence images for (i.) allantoic fluid, (ii.) QIV in allantoic fluid at 0.25 µg/mL, and (iii.) QIV at 0.25 µg/mL in PBS. (B) Quantification of HA in QIV vaccine spiked into PBS and allantoic fluid performed on three different days, with data from the three days represented by blue, orange, and purple open circles. Error bars represent the standard deviation for results from all three days combined. Abbreviations are as defined for Fig. 4.

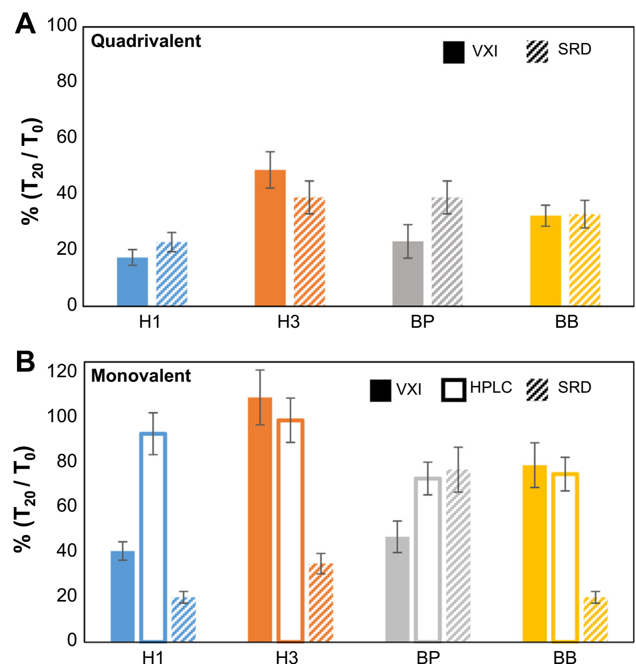

Fig. 6. Comparison of three methods for stability indication capabilities. Samples were heated at 56 °C for 20 h before analysis by VXI (solid bars), HPLC (open bars), or SRD (striped bars). Abbreviations are as defined for Fig. 4. Error bars are the standard deviations for triplicate VaxArray measurements and max validated method variability for HPLC and SRID measurements. (A) Quadrivalent vaccine. (B) Monovalent vaccines.

minor differences in formulation, storage conditions, and protein concentrations during thermal stress (e.g. monobulks tend to be at higher concentration).

The H3 antigen is of particular interest since by both VXI and HPLC it appeared to be the most stable antigen within both quadrivalent and monovalent vaccines, with no degradation detected by VXI within the monobulk sample under the applied stress conditions. For VXI, it is hypothesized that the anti-H3 capture monoclonal antibody binds to a particularly stable epitope for the HA protein and when used in combination with a polyclonal label the sensitivity of the assay to small perturbations in protein structure is reduced. HPLC measured concentrations for the monobulks show the H3 antigen to be the most stable and in general were more closely aligned with VXI values than with SRD values, as summarized in Fig. 6B. HPLC was not performed on quadrivalent vaccines.

Fig. 6 also shows the stability measurement performance of VXI relative to SRD, with error from triplicate measurements reported as standard deviation for VXI and validated method variability for SRD [31]. Within a quadrivalent vaccine, the %T0 values determined by VXI relative to the SRD %T0 values were 76%, 125%, 60%, and 98% for H1, H3, B/Phuket, and B/Brisbane, respectively. These ratios support a reasonable *qualitative* correlation between the two methods. However, for monobulks, the %T0 values determined by VXI relative to the SRD %T0 were 203%, 311%, 61%, and 394% for H1, H3, B/Phuket, and B/Brisbane, respectively. In contrast to VXI, the SRD assay indicated similar degradation for each antigen in both monobulks and quadrivalent vaccines, with the notable exception being B/Phuket. It is puzzling that for SRD measurements the B/Phuket stability was dramatically different in monobulk (77% T0) versus quadrivalent vaccine (39% T0) when this trend, which is consistent with that of VXI, was not observed for the other antigens. Additional work is needed to sort out the best stability testing configuration for VXI. For example, use of monoclonal label in VXI is anticipated to yield greater sensitivity to protein degradation. Ultimately, given the limitations of SRD, if differences arise in stability indication between SRD and an alternative potency assay it will be important to determine which assay better represents vaccine immunogenicity.

3.3.6. Next steps

Work is underway to understand the critical differences between traditional reference antigens, monobulk intermediates and final formulation vaccines. Other work includes a version of the microarray for application to potential pandemic vaccines, such as H5, H7, and H9 HA. In an early test of the VaxArray Pre-Pandemic Influenza potency assay, GSK provided two samples of H7/Shanghai vaccine for quantification. The samples were analyzed in triplicate and the average VXI-generated concentration values were 91 ± 5% of the SRD value (Fig. 4B), which is quite good agreement with SRD. The power of the VXI multiplexed platform is also being extended to achieve rapid and efficient quantification of neuraminidase.

4. Conclusions

This study demonstrated that the VaxArray potency assay exhibited good overall accuracy, precision, and stability indication properties when compared to SRD for both monobulk intermediates and multivalent split-virus flu vaccines. Relative to SRD, VXI exhibited a number of advantages, including speed (time to result 2 h vs. 48 h), off-the-shelf availability, wider linear dynamic range, greater sensitivity, and application to in-process samples. While additional work is needed to address the challenging issue of

appropriate reference antigens, this work establishes VXI as a promising alternative to SRD.

Potential conflict of interest

All authors are employed by their respective companies.

Authors' contributions

Dr. Kuck designed most of the experiments described in this study with Mr. Loob and Mr. Saye contributing execution of many of the experiments. Dr. Roth-Eichhorn designed the overall study and managed the experiments for HPLC and SDS-PAGE which were performed in GSK Quality Control labs. Dr. Rowlen invented the technology (patents pending) and provided scientific guidance on all work and Dr. Byrne-Nash contributed scientific review and preparation of the manuscript.

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