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Particle quantification of influenza viruses by high performance liquid chromatography

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Abstract

The influenza virus continuously undergoes antigenic evolution requiring manufacturing, validation and release of new seasonal vaccine lots to match new circulating strains. Although current production processes are well established for manufacturing seasonal inactivated influenza vaccines, significant limitations have been underlined in the case of pandemic outbreaks. The World Health Organization called for a global pandemic influenza vaccine action plan including the development of new technologies. A rapid and reliable method for the quantification of influenza total particles is crucially needed to support the development, improvement and validation of novel influenza vaccine manufacturing platforms. This work presents the development of an ion exchange-high performance liquid chromatography method for the quantification of influenza virus particles. The method was developed using sucrose cushion purified influenza viruses A and B produced in HEK 293 suspension cell cultures. The virus was eluted in 1.5 M NaCl salt with 20 mM Tris-HCl and 0.01% Zwittergent at pH 8.0. It was detected by native fluorescence and the total analysis time was 13.5 min. A linear response range was established between $1x10^9$ and $1x10^{11}$ virus particle per ml (VP/ml) with a correlation coefficient greater than 0.99. The limit of detection was between $2.07x10^8$ and $4.35x10^9$ whereas the limit of quantification was between $6.90x10^8$ and $1.45x10^{10}$ VP/ml, respectively. The coefficient of variation of the intra- and inter day precision of the method was less than 5% and 10%. The newly developed method was used to monitor virus concentrations in the supernatant obtained directly from the cell culture production vessels. HPLC data compared well with results obtained by HA assay. The HPLC influenza virus analytical method can potentially be suitable as an in-process monitoring tool to accelerate the development of processes for the manufacturing of influenza inactivated whole, split, subunit or live-attenuated vaccines.

Keywords (max 6): Influenza, HPLC, quantification, vaccine, particle, pandemic

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1. Introduction

Influenza, commonly known as "the flu" is a highly contagious viral infection of the respiratory tract caused by influenza viruses. Hemagluttinin (HA) and neuraminidase (NA) are the two major surface antigens that determine the subtype of the virus. Each year, approximately 500 million cases of influenza infection and between 250,000 to 500,000 deaths are due to seasonal epidemics [1]. Vaccination remains the most efficient strategy against influenza epidemics and pandemics. However, the manufacture of new vaccines is required due to frequent antigenic drift of the viral surface proteins. Since the 1950s, egg-based production processes remain the standard method to produce seasonal influenza inactivated whole, split, subunit or live-attenuated vaccines. However, the influenza 2009 H1N1 pandemic emphasized the limitations of this production method in a pandemic situation [2-4] since it takes 6 months from strain isolation to final dose formulation [5]. Furthermore, the World Health Organization (WHO) has established an action plan in 2006 to increase the current supply of influenza vaccine to 2 billion doses by 2015, which highlights the need to develop new technologies capable to support urgent and large demands for vaccines [6]. Therefore, several alternatives for rapid production have been developed or are being explored [7-12]. Some of these innovative production platforms only require 2 to 3 weeks to generate a new vaccine lot [13-15]. The bottleneck however is at the quantification level which can take weeks to months with the current traditional methods.

To assess the total influenza viral particle count in a preparation, transmission electron microscopy (TEM) is the only method to directly quantify influenza particles. However, it is costly, requires a high level of technical skills and works best with purified samples. Currently, an influenza vaccine dose is defined by the influenza HA protein content which can be established via the hemagglutination (HA) assay or other HA-related assay [16, 17], reverse transcriptase-polymerase chain reaction (RT-PCR) [18] and reverse phase (RP)-HPLC [19-22]. Officially however, only the single radial immunodiffusion (SRID) assay is approved by the regulatory agencies as a vaccine potency assay for lot release. The main limitation of the SRID assay is the requirement of influenza strain specific reference antigen/antibody. It is also a method that is highly subjective and therefore prone to variability. As a result, there is a consensus among the influenza vaccine community to develop alternatives to SRID potency assays. Consequently, rapid and reliable total particle quantification methods are of interest to facilitate and fasten the development of influenza vaccine manufacturing processes, and to monitor virus production. There have been a number of workshops convened by WHO and other vaccine organization to review the progress.

Ion exchange (IEX)-HPLC has been developed for the particle quantification of viral vectors for gene therapy and cancer therapy treatments produced in mammalian cell culture [23-28]. The use of this method in our group as an in-process monitoring tool was proven to be invaluable in the successful scale up of viral products that reached commercialization (ONRAB by Artemis Technologies, Canada) or are currently in late stage of clinical evaluation (Reolysin by Oncolytic Biotech., Canada). We demonstrate here that an IEX-HPLC method can be used for the quantification of total particle influenza virus from sucrose cushion purified samples as well as crude samples.

2. Materials and Methods

2.1. Cells, medium, influenza virus and production

The human embryonic kidney (HEK) 293 cell line, used for the production of the influenza viruses, was cultured in suspension in serum-free HyQSFM4 Transfx293™ (HyClone, USA). The influenza virus strains used in this study include A/Puerto Rico/8/1934 H1N1(PR8), A/Aichi/2/68 H3N2 (Aichi), A/Wisconsin/33 H1N1 (WS), A/Hong Kong/8/68 H3N2 (HK) and B/Lee/40 (B/Lee). The virus production was performed as previously described [9] at a Multiplicity of Infection (MOI) of 0.01 for all A strains and at an MOI of 0.1 for the B strain. Progeny viruses were collected at 48 hours post infection by centrifugation at 300g for 5 min.

2.2. Purification by ultracentrifugation in 25% sucrose cushion

The virus supernatant was clarified through a 1.0/0.45 µm Supor membrane (Pall Life Sciences, USA) followed by digestion with 30 U/ml of Benzonase (EMD Chemicals) for 30 min at 37°C. The clarified and digested supernatant was loaded into the ultracentrifuge tubes and underlayed with 25% sucrose cushion (in 20 mM Tris-HCl, pH 7.5) at a ratio of 10:1. Ultracentrifugation was performed at 37,000 x g for 3h at 4°C. The spent medium was discarded and the virus pellet was resuspended overnight at 4°C in 20 mM Tris-HCl +5% sucrose +2 mM MgCl2 at pH 7.5. The samples were then filtered through a 0.45 µm Supor membrane, aliquoted and stored at -80°C until further analyses. All viruses were concentrated 25 fold.

2.3. Negative stain electron microscopy

Negative stain electron microscopy (NSEM) was performed at Institut Armand Frappier (Laval, Canada) according to a method previously described [29]. The analysis was performed using two dilutions. The viral particle count was quantified as follows: VP/ml = (virus particle count/latex beads count) x (latex beads concentration*virus dilution).

2.4. Western blot analysis

SDS-PAGE was performed using a 4-15% Tris-HCl Mini-PROTEAN® TGX™ ready gels. To confirm the identity of the sucrose cushion purified viruses, western blot analysis was performed using a primary universal antibody against HA raised in rabbit [30]. Detection was performed using a secondary antibody infrared conjugated and the Odyssey scanner (LICOR Biosciences, Lincoln, USA). To confirm the location and identity of the flu peak in the HPLC analysis, material from several injections was collected and pooled; western blot analysis was performed using a sheep serum against A/PR/8/34 HA (NIBSC, UK).

2.5. Tissue culture infectious dose at 50% assay

The infectious titer of the viruses was quantified by a tissue culture infectious dose at 50% (TCID50) as previously described [9]. The cytopathic effects were revealed by alamar Blue [31].

2.6. Hemagglutination assay

Titration of the influenza viruses by HA was performed according to a method described previously [32] using red blood cells from 5 days old chicken at $2x10^7$ cells/ml (Charles River Laboratories, Canada). The HAU/ml obtained for all the viruses was converted to VP/ml using conversion factors as previously described [1].

2.7. HPLC conditions

The HPLC Alliance system (Waters, USA) equipped with a 2695 separations module, 996 photodiode array (PDA) detector, 2475 fluorescence detector and Empower™ software for data acquisition and integration was used. A CIMAc™ QA-0.1 monolithic analytical column (5.2 x 5.0 mm) (Canadian Life Science, Canada) was used to separate the virus. The mobile stock solutions were: A) 0.1 M Tris-HCl, pH 8 B) 2M NaCl, C) Milli Q® purified water and D) 1% Zwittergent 3-14 (Thermo Scientific). All mobile phases were prepared with Milli Q® purified water, filtered through a 0.45 ?m membrane and degassed for 10 min prior to use. The output stream from the column was monitored by native fluorescence (nFL) at the excitation and emission wave lengths of 290 nm and 335 nm, respectively.
The flow rate was kept at 1ml/min and all gradients were performed in 20% A. Samples were filtered through a 0.45 ?m Supor membrane (Pall Life Sciences, USA) before injection. The column was equilibrated with the start buffer (20%A, 0%B, 79%C and 1%D) for 10 min followed by 3 consecutive injections of 100 ?l buffer blank (BB, 20 mM Tris-HCl at pH 8). After sample injection, the gradient was started from 0 – 12.5% B for 2.5 min, and was held at 12.5% for 2 min. Next a step gradient at 75% B was performed to elute the virus and was held for 3 min. Finally, the column was re-equilibrated with the start buffer for 6 min before the next sample injection. For highly concentrated samples, a buffer blank was injected between each sample to minimize carryover. At the end of a sample set (maximum of 50 samples per set), 100 ?l 1M NaOH+1M KCl were injected 3 times to clean the column, followed by 3 injections of 100 ?l buffer blank. For persistent carryovers, the column was soaked with 1M NaOH overnight and rinsed off with Milli Q water.
The determination of the detection and quantification limit (DL and QL) was performed by 10 consecutive injections of a buffer blank (20 mM Tris-HCl, pH 8) to obtain a standard deviation (SD) of the response, which is also the background signal. DL and QL were calculated using the expression DL =3.3*SD/S and QL = 10*SD/S according to Q2B validation of analytical procedures published in 1996 by the FDA.

3. Results

3.1 Characterization of sucrose cushion purified viruses

The efficient production of five strains of influenza in HEK293 cells was first verified by western blot. All the viruses showed an HA0 band with an apparent molecular weight of 75 kDa, an HA2 band at 25 kDa and a putative truncated HA1 band at 37 kDa (Fig. 1A). Furthermore, the viruses produced are biologically active and infectious as determined in the TCID50 assay (Fig.1 B). Next, the viruses were characterized by NSEM. The presence of heterogeneous particle populations with various shapes and sizes was observed in all the samples (Fig. 1C). Most particles were of pleomorphic shape with the presence of spherical and filamentous particles. The average size obtained for PR8, Aichi, WS, HK and B Lee were 124±35 nm, 133±52 nm, 123±26 nm, 148±43 nm and 139±52 nm, respectively. Taken together, these results demonstrate that five strains of influenza virus with slightly different morphological characteristics were efficiently produced in HEK293 cells.

3.2 Quantification of sucrose cushion purified viruses

The total particle concentration of the sucrose cushion purified viruses was determined by NSEM and was compared to data obtained by HA assay. Overall, the same profile was observed with the two techniques for A/influenza subtypes (Fig. 1D). However, for WS and B Lee, the particle count obtained by the HA assay was significantly higher than NSEM by ~0.5 and 1.5 log, respectively.

3.3 Method development: virus separation, confirmation of virus peak and method specificity

Operation at pH 8 was selected based on the isoelectric point of most of the influenza viruses reported to be between 5.0 and 7.0 [33]. Preliminary work to determine the separation conditions used A/PR/8 as the model. The addition of 0.01% Zwittergent in the mobile phase was important to prevent non-specific ionic interactions due to the hydrophobic nature of the HA protein. Higher concentrations of the detergent were investigated but were detrimental to the particles. Several linear and step gradient methods were performed and found that the step elution at 1.5 M resulted in a single symmetrical peak (Figure 2B). This peak was confirmed to be the flu peak by WB analysis as shown by the presence of the HA1 and HA2 bands (Fig. 2B, inset).
The specificity of the method was determined by the analysis of a culture supernatant from a non-virus infected 293 cells and the cell culture medium used for production (Fig. 2C-D). With both samples, no interfering or co-eluting peaks were detected in the virus elution time (indicated by an arrow). Significant peaks were eluted at the beginning of the analysis and a peak eluted during the hold step at 250 mM in 1.87 min. Due to the high salt elution of the virus, double stranded (ds) DNA was also analyzed. Host DNA could be co-eluting with the virus because of similarities in charge and mass. The ds DNA standard did not show any peak (data not shown). These results demonstrate that the method is specific for the influenza virus particle elution and detection.

3.4 Analysis of other sucrose cushion purified influenza viruses and crude virus supernatants

Sucrose cushion purified samples of Aichi, WS, HK and B Lee were analyzed following the same protocol. For all the viruses tested, a single major peak eluting at 5.54 min and 1.5 M NaCl was detected (Fig. 3A-D) and attributed to the virus. Minor peaks that were well resolved from the virus peak were detected at the beginning of the analysis and at 250 mM NaCl gradient hold. Next, cell culture supernatants of the same viral strains were analyzed. These samples are less concentrated and contain more contaminants

than the sucrose-cushion purified samples. The virus peak was clearly detected and well resolved from the host-cell contaminant peaks that were mostly eluted at the beginning of the analysis and at the 250 mM NaCl gradient hold (Figure 3E-H).

3.5 Method qualification: Response signal difference among the viruses, quantification limit and precision

To quantify samples, a standard curve is generated based on the particle count obtained by NSEM. To determine if a universal standard could be used for all the influenza strains, equal concentration of all viruses was injected (9.5 $x10^8$ VP/ml) based on the particle count obtained by NSEM. As shown in Figure 4A the responses were different depending on the strain: the highest response is observed with B Lee, the lowest with PR8, while WS, Aichi and HK exhibit intermediate responses.
Given the response signal differences among the virus strains, the linearity of the method had to be determined independently for each influenza virus. The concentration measured by NSEM was used as the reference concentration. The relative SD of duplicate injections was less than 5% for all the standards (data not shown). Figure 4 shows that all the viruses had good linearity with a correlation coefficient greater than 0.99. The slopes were the same for WS and Aichi, HK was slightly lower, whereas B Lee had the steepest slope and PR8 had the least steep slope. These results reflect the differences in the fluorescence response signal obtained with B Lee being the most sensitive and PR 8 the least sensitive (Fig. 4). Next, the detection limit (DL) and quantification limit (QL) were determined. After 10 consecutive injections of a buffer blank (20 mM Tris-HCl, pH 8), the standard deviation (SD) of the response was 6.90 $x10^5$ VP/mL The DL and QL were calculated for each strain (Table 1).
Finally, the coefficient of variation of the intra-day assay precision or repeatability of the method was less than 5% for all the viruses except for PR8, which was less than 10% (Table 1). The coefficient of variation of the inter-day assay precision, which reflects the day-to-day variability, was less than 10% except for PR8, which was less than 20% after injecting for three consecutive days (Table 1). The coefficients of variation of the intra- and inter-day precisions for the retention time were less than 2% for all the virus strains and subtypes (data not shown).

3.6 On-line monitoring of influenza virus production samples collected in the cell culture supernatants

The applicability of the method to directly quantify the influenza virus particle from cell culture supernatant was assessed by collecting samples of influenza A virus PR8 at different hours post infection (hpi) during a bioreactor production run (Fig. 5A). The linear curve previously generated for PR8 was used for the quantification. The expected profile was obtained with a steady increase between 24 hpi and 48hpi. Next, samples from different productions in shake flasks were quantified by HPLC and compared to results obtained by HA (Fig 5B). A good correlation was observed between the two methods even though the particle count tends to be higher when measured by the HA assay for the two H3N2 subtypes (Aichi and HK). It should be noted that H3N2 particles possess about 40% more glycoproteins on their surface than H1N1 strains which could explain this discrepancy [34].

4.0. Discussion

The HPLC method described here is based on the measure of intrinsic or native fluorescence (nFL) of proteins, mostly due to the three aromatic amino acids (tryptophan, tyrosine and phenylalanine) when excited at wavelengths between 280 and 295 nm. Tryptophan contributes to the majority of the signal because it has the highest quantum yield among the three residues. It has been demonstrated that the HA1 detection of influenza vaccines was significantly improved by up to 2.7 logs when using nFL instead of the classical 280 nm UV absorbance [19, 21].

The results presented in figure 3 support the suitability of the method for the analysis of influenza strains B and A (subtypes H1N1 and H3N2), and its potential for generalization to all other influenza strains and subtypes. Most importantly, the method is appropriate to separate, identify and detect the virus in the cell culture supernatants allowing a potential in-process analysis of influenza virus production in real time. However, we observed a differential response depending on the strains (Fig. 4) that could be explained by differences in morphology, glycoprotein density on the surface and amino acid composition of each strain. These differences have been observed with other quantification methods based on physical characteristics of the particle. For instance, the particle count obtained for B/Lee by the HA assay was 1.5 log higher than by NSEM which could be due to higher agglutination activity of B/Lee viruses [35]. Also, the amino acid (a.a.) composition of the different HA molecules (Suppl. Table 1) would affect the detection signal. The HA total number of amino acids (a.a.) is highest with B Lee (584 a.a.), lowest for PR8 (565 a.a.) and intermediate for Aichi and WS (566 a.a.). Precise quantification will therefore require the generation of a standard curve for each different strain.

The accelerated development of improved or novel influenza candidate vaccines requires rapid and reliable quantification methods to support process intensification, and in-process monitoring and control techniques. Current quantification techniques are generally cumbersome with a response time of days or weeks [36]. Therefore, IEX-HPLC is one of the most promising technologies for total viral particle determination. In the case of live attenuated or inactivated influenza vaccine candidates accessing within minutes the viral particle concentration at all steps of the manufacturing process would be an important progress to support the acceleration of the process development and an important process analytical tool for the quality by design of the industrial process.

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