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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 1×10^9 and 1×10^{11} virus particle per ml (VP/ml) with a correlation coefficient greater than 0.99. The limit of detection was between 2.07×10^8 and 4.35×10^9 whereas the limit of quantification was between 6.90×10^8 and 1.45×10^{10} VP/ml, respectively. The coefficient of variation of the intra- and inter-day precision of the method was less than 5% and 10%. HPLC data compared well with results obtained by electron microscopy, HA assay and with a virus counter, and was used to monitor virus concentrations in the supernatant obtained directly from the cell culture production vessels. 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 vaccines.

1. Introduction

Influenza, commonly known as "the flu" is a highly contagious viral infection of the respiratory tract caused by influenza viruses. Hemagglutinin (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 and 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 2–3 weeks to

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generate a new vaccine lot [13–15]. The bottleneck however is at the quantification level which can take weeks to months with the current methods. The Single Radial Immunodiffusion assay (SRID) is the sole approved quantification method for influenza vaccine release. In addition to the long delay for the generation of specific antibodies and reference antigens necessary to perform the assay, the method is very time-consuming, labor intensive and imprecise.

Transmission electron microscopy (TEM) is traditionally used to directly assess the total influenza particle concentration in a virus preparation. However, it is costly, requires a high level of technical skills and works best with purified samples. New technologies have also been developed to measure the particle count of viruses such as ViroCyt and NanoSight® devices but these equipment are not readily available in most laboratories. A recent review summarizes the main available techniques to quantify viral particles [16]. Consequently, rapid, reliable and easily accessible total particle quantification methods are of interest to facilitate and expedite 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 [17–22]. 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). In this manuscript, we demonstrate that an IEX-HPLC method can be used for the quantification of influenza virus particle using sucrose cushion (SC) purified and virus supernatant samples. The quantification is based on the native fluorescence (nFL) detection of the virus proteins using a calibration curve generated from an in-house virus standard with a TEM pre-determined particle concentration.

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), A/Aichi/2/1968 (H3N2), A/Wilson-Smith/1933 (H1N1), A/Hong Kong/8/1968 (H3N2) and B/Lee/1940, and they are abbreviated PR8, Aichi, WS, HK and B/Lee respectively throughout the text. 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 h post infection by centrifugation at 300 × *g* 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% SC (in 20 mM Tris–HCl, pH 7.5) at a ratio of 10:1. Ultracentrifugation was performed at 37,000 × *g* for 3 h 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 $MgCl_2$ 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 [23]. The analysis was performed using two dilutions. The viral particle count was quantified as follows: VP/ml = (virus particle count/latex beads count) × (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 SC purified viruses, Western blot (WB) analysis was performed using a primary universal antibody against HA raised in rabbit [24]. 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; WB 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 (IVP/ml) of the viruses was quantified by the tissue culture infectious dose at 50% (TCID50) assay as previously described [9]. The cytopathic effects were revealed by alamar Blue (Life Technologies) [25].

2.6. Hemagglutination assay

Titration of the influenza viruses by HA was performed according to a method described previously [26] using red blood cells from 5 days old chicken at 2×10^7 cells/ml (Charles River Laboratories, Canada). The HAU/ml obtained for A/Puerto Rico/8/1934 H1N1(PR8), was converted to VP/ml using conversion factors as previously described [1,27].

2.7. Viral counter

Influenza particles were quantified with a Virus Counter 3100 (ViroCyt, Colorado, USA) using a double staining method according to the manufacturer's recommendation. A demonstration instrument was kindly provided by the manufacturer for performance of the analyses on NRC Montreal site.

2.8. 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 mm × 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) 2 M 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 μm 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 1 ml/min and all gradients were performed in 20% A. Samples were filtered through a 0.45 μm 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 three consecutive injections of 100 μl buffer