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Accelerated mass production of influenza virus seed stocks in HEK-293 suspension cell cultures by reverse genetics

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ABSTRACT

Despite major advances in developing capacities and alternative technologies to egg-based production of influenza vaccines, responsiveness to an influenza pandemic threat is limited by the time it takes to generate a Candidate Vaccine Virus (CVV) as reported by the 2015 WHO Informal Consultation report titled "Influenza Vaccine Response during the Start of a Pandemic".

In previous work, we have shown that HEK-293 cell culture in suspension and serum free medium is an efficient production platform for cell culture manufacturing of influenza candidate vaccines. This report, took advantage of, recombinant DNA technology using Reverse Genetics of influenza strains, and advances in the large-scale transfection of suspension cultured HEK-293 cells. We demonstrate the efficient generation of H1N1 with the PR8 backbone reassortant under controlled bioreactor conditions in two sequential steps (transfection/rescue and infection/production). This approach could deliver a CVV for influenza vaccine manufacturing within two-weeks, starting from HA and NA pandemic sequences. Furthermore, the scalability of the transfection technology combined with the HEK-293 platform has been extensively demonstrated at >100 L scale for several biologics, including recombinant viruses.

Thus, this innovative approach is better suited to rationally engineer and mass produce influenza CVV within significantly shorter timelines to enable an effective global response in pandemic situations.

1. Introduction

The influenza virus is responsible for a global epidemic every year that infects millions of people and causes serious illness and death worldwide [1]. Vaccination remains the primary and most effective strategy for the prevention and control of influenza. Due to the high mutation rate of the influenza virus, vaccine manufacturers must reformulate their products every year to ensure a good match between the HA and NA present in the vaccine and the circulating strains [2]. The majority of the current licensed influenza vaccines are made using embryonated hen's eggs, a system that has been used for more than 60 years [3]. Increased demand and the threat of a pandemic outbreak have accelerated the introduction of new manufacturing strategies for influenza vaccine production. The US Human Health Services actively supported the development of alternative manufacturing strategies for influenza vaccine. For example, cell-based production technology allows manufacturers to respond to public health needs faster and in shorter production cycles. It also allows for greater surge capacity, greater process control and more reliable and well-characterized products [4–6]. Yet in spite of major advancements and licensing of cell culture manufactured processes [7], responsiveness to an influenza pandemic threat remains limited by the timely availability of the "master viral seed stock" or Candidate Vaccine Virus (CVV) as reported in the last WHO Informal Consultation report 2015 titled "Influenza Vaccine Response during the Start of a Pandemic" [8].

Reverse genetics can be used to streamline the generation of viral seed stocks, which would be vital in case of a pandemic outbreak, thereby avoiding the long and cumbersome reassortant generation process in eggs [2,9]. Reverse genetics is a process to generate live viruses from a set of plasmid-cloned cDNA collectively encoding the influenza viral genome [10,11]. This strategy can be used to generate specific and high-growth 6 + 2 reassortant

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viruses integrating only the HA and NA gene segments of the seasonal strain within the Puerto Rico backbone for further use as viral seeds in egg-based or cell culture technology [12]. However, all current protocols for obtaining influenza viral particles by reverse genetics use a co-culture of adherent cells [13], such as HEK293T, COS-1, Vero, CHOK1 or MDCK [14–16], limiting the scalability of the system. The use of suspension cell lines such as HEK-293 improves the scalability of the process, which is a key element in large-scale cell culture vaccine production [17,18]. Building on extensive developments and expertise from our group and others in large-scale transient transfection using HEK-293 suspension cultures to produce RNA and DNA recombinant viral vectors [19–23], we demonstrate that influenza PR8 can be effectively produced by reverse genetics from transfected suspension HEK-293 cells at both small and large-scale after only one amplification step. Moreover, the characterization of produced virions showed no change of the viral antigen sequences Overall, we propose that HEK-293 suspension cells can be used as a production platform for influenza A viruses as an alternative and rapid system to obtain reassortant viral seed stocks (CVV) for vaccine manufacture.

2. Materials and methods

2.1. Cell lines and culture conditions in small and large scale

The human embryonic kidney (HEK-293SF) cells adapted to suspension and derived from a cGMP master cell bank were cultured in serum-free SFM4Transfx-293™ (HyClone, USA) supplemented with 4 mM of L-glutamine (HyClone, USA) in a humidified incubator at 37 °C with 5% CO_2, at an agitation rate of 100–110 rpm. MDCK cells were cultured in EMEM medium (ATCC, USA) supplemented with 10% (v/v) Fetal Bovine Serum (NorthBio, Canada) in a humidified incubator at 37 °C with 5% CO_2.

For large-scale experiments, a 3-L Chemap type SG bioreactor (Mannedorf, Switzerland) was used with a working volume of 1.8 L at an initial concentration of 0.16×10^6 cells/mL. The bioreactor features were published in previous publication of our group [24]. Cells were grown for 72 h until reaching a density of $\sim 1.0 \times 10^6$ cells/mL for transfection. Samples were taken once a day for cell culture and viral production monitoring.

2.2. Transient transfection

2.2.1. Plasmids

The cDNA obtained from the different genes of the virus A/Puerto Rico/8/34 (H1N1) were cloned into the pHW2000 vector [14]. Briefly, viral RNA was extracted by using QIAmp viral RNA minikit (Qiagen) from infected-MDCK cell culture supernatant, according to the manufacturer's instructions. Two-step RT-PCR was carried out for full-length amplification of each viral RNA gene segment, by using influenza A universal RT primers (Uni-12primer «3′-AGCAAAAGCAGG-5′», Eurogentec, Belgium) as previously described [25].

The eight plasmids [pHW2000-PB1, pHW2000-PB2, pHW2000-PA, pHW2000-NA, pHW2000-M, pHW2000-NS, pHW2000-HA and pHW2000-NP] were amplified using the bacterial strain *E. coli* TOP10 (Invitrogen, USA) and purified using the GigaPrep Extraction Kit (Macherey Nagel, USA). Plasmid DNA quantity was assessed by NanoDrop (Thermo Scientific, USA). The viral sequences within the eight plasmids were controlled and validated by sequencing by MWG Eurofins Company.

2.2.2. Transfections in shake flasks

Suspension HEK-293 cells at 1×10^6 cells/mL were transfected with 25-kDa linear polyethylenimine (PEIpro) (PolySciences, USA) in 20 mL working volume shake flasks. Complexes were generated by mixing 1 μg of total plasmid DNA/mL cell culture and 2 μg PEIpro/mL cell culture. Mixture was vortexed for 5 s and incubated at room temperature for 10–15 min. After incubation the volume of transfection was adjusted to 10% total volume with medium and transferred drop-by-drop into the shake flasks. The flasks were incubated at 37 °C and 5% CO_2. After 24 h post transfection (hpt), 1 μg/mL TPCK-Trypsin (Affymetrix, USA) was added. The viral particles were harvested at 48 hpt generating the P0 of the influenza A/Puerto Rico/8/34 virus. For the P0 amplification and generation of P1, 10 mL of the supernatant of P0 were used to infect 10 mL of suspension HEK-293 cells at 2×10^6 cells/mL in medium with 1 μg/mL TPCK-Trypsin (final concentration). The infected flasks were incubated for 96 h post-infection (hpi).

2.2.3. Transfection in 3-L bioreactor scale

Cells at 1×10^6 cells/mL (1.8 L) were transfected using 200 mL (10% of the final volume) of medium, PEIpro and plasmid DNA for a final volume of 2L in the bioreactor. Complexes were generated as described above. The mixture was transferred into the bioreactor by gravity using the inoculum bottle. After 24 hpt, 1 μg/mL TPCK-Trypsin (final concentration) was added into the bioreactor. After 48 hpt the bioreactor was harvested (P0) and used to infect 20 mL suspension HEK-293 (2×10^6 cells/mL) in shake flask to generate the P1 following the protocol described above (Fig. 1). Table 1 summarizes the main conditions used in this work for cell transfection in shake flasks and bioreactor.

2.3. Influenza virus quantification and characterization

2.3.1. Tissue culture infectious dose at 50% assay ($TCID_{50}$)

The infectious titer (IVP/ml) of the viruses was quantified by the tissue culture infectious dose at 50% ($TCID_{50}$) assay as previously described [24]. The cytopathic effects were measured using Alamar blue (Life Technologies) [26].

2.3.2. Dot Blot assay

The protocol followed in this study for hemagglutinin (HA) and neuraminidase (NA) has been previously published [27]. Briefly, a calibration curve was generated using a recombinant protein for NA (H1N1/A/USSR/90/77 from Sino Biological Inc., China) and an in-house standard for HA PR8 virus semi-purified by sucrose cushion and previously quantified by SRID. The primary antibody used for the dot-blot assay against HA was an anti-HA monoclonal antibody produced in-house; the membranes were probed with 6 μg/ml anti-HA antibody overnight at 4 °C. For the dot-blot against NA, a universal rabbit anti-NA HCA-2 antibody was kindly provided by Health Canada [28]. Infrared-conjugated secondary antibodies (LI-COR Bioscience, USA) were used to reveal the signal with an Odyssey CLx imaging system (LI-COR Bioscience, USA).

2.3.3. Single radial immunodiffusion assay

A previously published protocol [29] was used. In absence of the final calibration of antigen reagent 14/200 from NIBSC at the time of the study, an in-house reference material using an influenza A PR8 sucrose cushion stock extensively characterized was used for the standard curve. The antibody used to form precipitation rings was a sheep polyclonal antibody (Ref. 03/242, NIBSC, UK).

2.3.4. Hemagglutination assay

The protocol for hemagglutination (HA) assay described previously was used to determine the quantity of Total Viral Particles (VP)/ml [29,30].

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