Influenza B virus reverse genetic backbones with improved growth properties in the EB66<sup>®</sup> cell line as basis for vaccine seed virus generation

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**Abstract**

Vaccination remains the best available prophylaxis to prevent influenza virus infections, yet current inadequacies in influenza virus vaccine manufacturing often lead to vaccine shortages at times when the vaccine is most needed, as it was the case during the last influenza virus pandemic. Novel influenza virus vaccine production systems will be crucial to improve public health and safety. Here we report the optimization of influenza B virus growth in the proprietary EB66<sup>®</sup> cell line, currently in use for human vaccine production. To this end, we collected, curated and sequenced 71 influenza B viruses selected for high diversity in date of isolation and lineage. This viral collection was tested for ability to enter and replicate within EB66<sup>®</sup> cells in a single cycle assay and appears to readily infect these cells. When the collection was tested for viral progeny production in a multi-cycle assay, we found a large variation from strain to strain. The strains with the top growth characteristics from the B/Victoria and B/Yamagata lineages were selected for vaccine backbone generation using a reverse genetics system. We then showed that these backbones maintain their desirable growth within EB66<sup>®</sup> cells when the HA and NA from poorly growing strains were substituted for the parental segments, indicating that the selected backbones are viable options for vaccine production in EB66<sup>®</sup>. Finally, we show that compounds previously reported to enhance influenza virus growth in cell culture also increase virus production in the EB66<sup>®</sup> cell line.

1. Introduction

Influenza virus is an important human pathogen which causes significant economic loss, morbidity, and mortality in annual epidemics worldwide [1–3]. In addition to annual epidemics, pandemics of antigenically distinct influenza viruses occur periodically with outcomes ranging from mild to devastating to the human population. The “Spanish Flu” pandemic of 1918 was recorded as the worst influenza pandemic ever and one of the worst infectious disease events in human history, killing an estimated 50–100 million people [4–6].

Since 1918, there have been major advances in influenza virus surveillance, antivirals and vaccines for detection and mitigation of potential pandemics. Most recently, the H1N1 pandemic of 2009 was a test of our modern national pandemic preparedness system. The pandemic H1N1 virus was detected in March of 2009, by April the CDC had begun developing vaccine seed candidates for the novel swine origin H1N1 influenza virus, and large-scale vaccine production in embryonated chicken eggs began in late June. Upon FDA approval of the pandemic vaccine, production and delivery fell short of public need during the peak of the pandemic in late October, achieving less than 20% population coverage in the US by the end of December [27]. This shortfall of delivered pandemic influenza virus vaccine in 2009, combined with the possibility of embryonated egg shortages, highlights a glaring need for new manufacturing strategies with improved speed in vaccine lead and production times, allowing continued supply of the needed vaccines to the population [7,8].

Novel cell culture systems have the potential to offer multiple advantages for future vaccine production. Most prominently, optimized cell-based systems have the potential to achieve faster lead/production times with more vaccines delivered [9–11]. This increased manufacturing capacity would also have the benefit of stability, not being reliant on the availability of embryonated chicken eggs. Additionally, vaccines produced in cell culture...
systems have higher similarity to circulating strains and do not suffer from allergic reactions to egg components [12,13]. While recombinant HA-based vaccines provide a cell-based alternative to inactivated influenza virus vaccines grown in eggs [14], manufacturing of live and/or inactivated influenza virus vaccines in tissue culture requires suitable cells and viral strains able to support replication of the influenza vaccine virus to high titers.

The duck embryo-derived EB66<sup>®</sup> cell line is susceptible to infection by a broad range of different viruses, including influenza viruses and has been shown to have features conducive to industrial vaccine manufacturing [15–18]. In fact, it is in use to produce an H5N1 influenza virus vaccine which has been approved for marketing by the Japanese health authorities [16]. Optimization of growth of influenza viruses in EB66<sup>®</sup> cells has been achieved through development of culture media and manufacturing processes leading to robust viral production [19]. In addition, viral growth improvements might also be achieved by selecting for high yield viral backbones supporting high levels of replication of antigenically relevant vaccine strains [20]. Here we report the evaluation of the EB66<sup>®</sup> cell line as a production system for the influenza B component of a seasonal influenza virus vaccine. We found that while the influenza A/PR/8/34 virus, commonly used as the backbone virus for inactivated H1N1 and H3N2 influenza A virus vaccine strains, grew to high titers on this cell substrate, several influenza B virus strains produced significantly lower yields. We tested a library of 71 influenza B viruses and found large strain-to-strain variation within the group. We selected the top yielding strains based on hemagglutination titers from both the Yamagata and Victoria lineages and generated reverse genetic systems. We demonstrated that these backbones maintain their high-growth phenotype when reassorted with the HA and NA from poor growing strains, indicating that they are potentially viable for vaccine virus generation. Finally, we show that influenza virus yields from EB66<sup>®</sup> can also be increased with known influenza virus growth enhancing compounds.

2. Material and methods

2.1. Cell culture and reagents

Madin-Darby canine kidney (MDCK) epithelial cells and human embryonic kidney 293 T (293 T) cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). EB66 cells were provided by GlaxoSmithKline (GSK, Wavre, Belgium) through a licensing agreement with Valneva (Lyon, France). MDCK and 293 T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT), and 1% penicillin-streptomycin (P/S) (Gibco) and grown at 37 °C, 5% CO<sub>2</sub>. EB66 cells were cultured in DC02 media co-developed by SAFC/Valneva and formulated by GSK at 37 °C, 7.5% CO<sub>2</sub>.

2.2. Viral stocks and full genome sequencing

Influenza B strains were collected from samples stored at the Department of Microbiology at the Icahn School of Medicine at Mount Sinai (ISMMS), as well as from the American Type Culture Collection (ATCC), Centers for Disease Control (CDC), Influenza Reagent Resource (IRR), Biodefense and Emerging Infections Research Resources Repository (BEI) and National Institute for Biological Standards and Control (NIBSC). Strain identification numbers, when available, are listed in Table 1. All influenza B virus strains were propagated in 8-day old embryonated chicken eggs for 3 days at 33 °C. The control influenza A strain A/Puerto Rico/8/34 (PR8) was propagated in eggs for 2 days at 37 °C. Viral stocks were aliquoted from the harvested allantoic fluid and titrated by standard plaque assay in MDCK cells. Mouse-adapted variants of B/Malaysia/2506/2004 and B/Florida/4/2006 were generated before the current gain-of-function studies moratorium and have been described elsewhere [21,22].

Viral RNA was purified from viral stocks using the E.Z.N.A. Viral RNA kit (Omega bio-tek) following manufacturer’s instructions, and used to determine consensus full genome sequences as follows: RNA-Seq libraries were prepared using 1 μg of total viral RNA. The TruSeq RNA Library Preparation Kit v2 was used according to the manufacturer’s instructions. Briefly, RNA was purified via random hexamer beads, fragmented, and reverse transcribed with SuperScript II (Invitrogen), followed by second strand synthesis and adapter ligation. Quantification of barcoded samples and pooled libraries were assessed using the Universal Complete KAPA Library Quantification Kit (KAPA Biosystems). Pooled libraries were run on an Illumina MiSeq platform using the MiSeq Reagent Kit V3 (Illumina). Read mapping, statistical analysis, and alignments were performed using Bowtie2 and Integrated Genome Viewer (iGV). Consensus sequences were aligned and analyzed with Clustal OMEGA (EMBL-EBI). Phylogenetic trees were generated with FigTree v1.4.3 (University of Edinburgh).

2.3. Viral infectivity assays

25,000 EB66 cells were infected in suspension with various influenza B virus strains to achieve an MOI of 0.01, 0.1 or 1 in 100 μl of DC02 media (co-developed by SAFC/Valneva and formulated by GSK) without trypsin in 96-well format. EB66 cells attached to plates and infections were allowed to proceed for 16 h at 33 °C, 5% CO<sub>2</sub> followed by fixation with formaldehyde. Cells were blocked with 1% BSA for 30 min and were then incubated for 1 h with a cocktail of murine monoclonal antibodies specific to the influenza B virus HA protein produced by Teddy John Wohlbold from his unpublished collection. Cells were then incubated with an AlexaFluor 488 (Invitrogen) secondary antibody (dilution 1:1000) for 1 h with 10 μg/ml ethidium bromide as a cellular counterstain. Influenza B virus infected cells were detected by laser scanning cytometry (Acumen, TTP Labtech) and infectivity determined by 488 nm signal over counterstain of total cells.

2.4. Viral growth assays

2 × 10<sup>6</sup> EB66 cells were infected in suspension with various influenza B virus strains to achieve an MOI of 0.01 in DC02 media (co-developed by SAFC/Valneva and formulated by GSK containing 1 μg/mL tosyl phenylalanyl chloromethyl ketone (TPCK) –treated trypsin (Sigma-Aldrich, St. Louis, MO) in 12-well plates. Infected samples were incubated while continuously shaking at 150 rpm for 72 h at 33 °C, 7.5% CO<sub>2</sub>. Plates were covered with Aeroseal film (Sigma-Aldrich, St. Louis, MO) to prevent evaporation and cross-contamination. Viral titers were determined by standard plaque assay in MDCK cells.

2.5. Hemagglutination assay

Infected samples were harvested and centrifuged at 5000 RPM × 3 min to clear the supernatant of cells and debris. 50 μl of clarified supernatants and 11 serial 2-fold dilutions were then mixed with 50 μl of 0.5% of chicken red blood cells in V-bottom 96-well plates. Plates were incubated at 4 °C for 30 min and HA titer determined by the reciprocal of the last dilution which showed complete hemagglutination.
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