STABILITY EVALUATION OF INACTIVATED INFLUENZA H7N9 VACCINES DERIVED FROM ADHESION AND SUSPENSION MDCK CELLS

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Key word: antigen stability, *suspension MDCK* cells, chemical-defined medium, H7N9 virus

In recent years, cell-based manufacturing processes for influenza vaccine production has gained a great interest over the egg-based process. We have adapted MDCK cells to grow in chemical-defined medium and found this newly suspension MDCK cell line (sMDCK cells) is very suitable for the production of influenza virus. However, the property of purified antigens from sMDCK cells remains unclear. In this study, a stability program of influenza H7N9 vaccine (NIBRG268 vaccine strain) produced by sMDCK cells was investigated, and the data were compared with the vaccine derived from adhesion MDCK (aMDCK) cells in serum-free medium. The H7N9 bulks (with different storage time) derived from sMDCK and aMDCK cells were stored at 2-8°C for some times, and a number of parameters were used to monitor the H7N9 vaccine antigen stability was evaluated at different periods (1, 2, 3 and 6 months). The monitoring parameters are including virus structure, HA titer, HA content, total protein level, antigenicity, and immunogenicity. The sMDCK-derived H7N9 bulk showed similar virus structure to that aMDCK-derived H7N9 bulk, and there was no obvious change after further 6 months of storage. Furthermore, HA titer, HA content and total protein level of sMDCK- and aMDCK-derived H7N9 bulks were stable after 6 months of storage. sMDCK- and aMDCK-derived H7N9 bulks displayed similar antigenicity detected by hemagglutination inhibition (HI) test using standard serum. Finally, the results of HI and neutralization tests showed that sMDCK- and aMDCK-derived H7N9 vaccines were similar in immunogenicity in BALB/c mice vaccinated with 0.2 μg of H7N9 vaccine with an adjuvant of aluminum hydroxide. These results indicate that sMDCK-derived H7N9 bulk has good stability data similar to the aMDCK-derived H7N9 bulk. Thus, the newly developed suspension MDCK cells show a great market potential over the traditional vaccine manufacturing methods.

RAPID, COST-EFFECTIVE AND SCALABLE GMP-COMPLIANT SIMIAN ADENOVIRUS-VECTORED VACCINE PRODUCTION FOR EARLY-PHASE CLINICAL TRIALS USING ENTIRELY DISPOSABLE PRODUCT-CONTACT COMPONENTS

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Key Words: Simian adenovirus, GMP, clinical trials, single-use, biomanufacturing

The Jenner Institute, University of Oxford, develops and produces a range of vaccines against emerging threats (such as Zika) and current global health challenges (including malaria, HIV and rabies). The Jenner Clinical Biomanufacturing Facility (CBF) manufactures multiple simian adenovirus-vectored vaccines for early phase clinical trials each year. Hitherto we have used shake flasks for upstream production and caesium chloride gradient ultracentrifugation for downstream purification. This process is robust and simple but also slow, human resource intensive and lacks scalability.

Here we report the development of a novel process using a 2 x 3L single-use stirred tank bioreactor system (MilliporeSigma Mobius®), coupled to a tangential flow filtration (TFF) and anion exchange chromatography (AEX)-based downstream process. The process also includes particle lysis and nucleic acid digestion inside the bioreactor, as well as clarification of cells and debris using depth filters. As our test case, we used a novel simian adenovirus-vectored rabies vaccine (ChAdOx2 RabG), which we will manufacture to GMP standards in the coming year. Each process run yields >$5x10^{13}$ ChAdOx2 RabG virus particles (approximately 1000 human doses), with residual host cell DNA, host cell protein and nuclease levels suitable for clinical trial use.
While similar processes have been previously reported for adenovirus manufacture, we will report a number of points of novelty. Firstly, we use single-use disposable product-contact components from beginning to end, greatly simplifying small-scale GMP manufacturing of multiple products. Secondly, we will report results of comparative testing with a range of modern ion exchange media (including resins, membrane adsorbers, monoliths and functionalized hydrogel formats). Thirdly, we will report the development and validation of novel quality control methods suitable for this process.

The resulting process will allow the CBF to increase production yield and produce more vaccines that transfer more easily to larger facilities.

FLAVIVIRUS PRODUCTION IN PERFUSION PROCESSES USING THE EB66® CELL LINE

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Key Words: EB66®, flavivirus, perfusion, on-line capacitance, cryo-bag.

The outbreak of mosquito-borne yellow fever virus (YFV) in Angola 2016 rapidly spread to urban regions and other countries. Vaccination campaigns were subsequently intensified, but the increased vaccine demand led to depleted stockpiles. Current yellow fever vaccine manufacturing processes rely on embryonated chicken eggs, which are strongly limited with respect to flexible capacity increase in emergencies. The global vaccine demand is estimated by the WHO to 1.38 billion doses needed to eliminate epidemics. Thus, an urgent need for an improved production platform is needed, ideally transferable to new vaccine developments against emerging flaviviruses, such as Zika virus.

Here we present a cell culture-based YFV 17D and Zika virus (ZIKV) production process using the EB66® cell line. The avian EB66® suspension cell line grew fast and stable in chemically defined medium to cell concentrations of 1.8×10^7 cells/mL in shake flasks and batch mode. Seed virus was prepared from Vero-derived YFV and ZIKV material over five passages in EB66® cells. Thereby, infectious virus titers successfully increased by one log unit and maximum titers of 1.4×10^8 PFU/mL (infectious virions per mL) and 8.0×10^7 PFU/mL were obtained two days post infection for YFV and ZIKV, respectively.
The process was intensified using perfusion bioreactors to increase cell concentrations. Therefore, EB66® cells were cultivated in 1 L benchtop bioreactors equipped with an alternating tangential flow filtration (ATF2) perfusion unit. Perfusion rates were adjusted to maintain glutamine concentrations above 1 mM and cells grew up to 9.5×10^7 cells/mL. A maximum YFV titer of 7.3×10^8 PFU/mL was achieved. The cell-specific virus yield (CSVY) was 8 PFU/cell, similar to shake flask experiments.

For ZIKV production, another approach aimed at the use of on-line capacitance sensors to control cell-specific perfusion rates (CSPRs) based on cell concentrations. This automated system was set to a CSPR of 0.017 and 0.034 nL/cell/day leading to maximum cell concentrations of 8.9×10^7 cells/mL and 1.6×10^8 cells/mL. ZIKV titers peaked after three to four days post infection with 2.6×10^9 PFU/mL and 1.0×10^{10} PFU/mL, respectively. CSVYs increased from 5 PFU/cell (shake flask experiments) to 30 PFU/cell and even above 64 PFU/cell in this set-up. The increased CSPR resulted in an improved volumetric productivity by factor three compared to the lower CSPR.

Further process intensification was achieved by direct cell inoculation to the ZIKV production bioreactor. A 15 mL cryo-bag was thawed with 8.5×10^8 cells and cell viabilities of 90% after inoculation quickly increased over the cultivation period.

Taken together, EB66® suspension cells can be grown to concentrations exceeding 1.5×10^8 cells/mL in perfusion bioreactors, and cells are highly permissive for YFV and ZIKV. YFV production using perfusion systems generated virus material equivalent to 10 Mio vaccine doses (4.7 log infectious units per dose) in less than two weeks operation time. With the use of on-line sensors to adjust CSPRs meeting cellular nutrient demands, ZIKV titers exceeding 1.0×10^{10} PFU/mL were obtained for the first time. Direct cryo-bag inoculation shortened the seed train phase, and virus production was initiated with full flexibility. This is a powerful demonstration on how next generation flavivirus vaccine production can be realized.

LATEST ADVANCEMENTS IN PROCESS INTENSIFICATION TO SUPPORT GLOBAL DEMAND FOR AFFORDABLE VACCINES

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Vaccines have a profound impact on global health, preventing illness, death, and improving the quality of life across the globe. However, the current costs of vaccine manufacturing and distribution often prevent the poorest segments of the world's population from accessing these critical medicines.

Vaccine manufacturing for global distribution typically requires large and expensive manufacturing facilities that result in high vaccine Cost of Goods and impede developing countries from initiating or expanding in-country manufacturing capabilities.

One of the strategies to address this is to intensify vaccine production processes. This presentation will give an overview of a project funded by the Bill & Melinda Gates foundation that focusses on applying the latest process intensification technologies to develop a platform that dramatically increases process efficiency, decreases production scale and reduces vaccine COGs to a price of $0.15 per dose or less.
This vaccine manufacturing platform combines Vero cell lines optimized for virus propagation and media capable of supporting high cell density cell growth, high cell density single-use bioreactors and high efficiency and single step purification technologies.

Together, these technologies enable vaccine yields to be significantly increased, which in turn allows commercial manufacturing in a small-footprint, low cost "micro-facility" capable of delivering >40M doses of vaccine per year for a CAPEX of not more than $10M and low operational costs. Such "micro-facilities" can be rapidly and inexpensively commissioned, drastically reducing vaccine COGs, facilitating rapid response and resulting in commercial manufacturing at lab scale.

The platform is currently being established using Sabin IPV as the target vaccine. Current status is that Vero cell lines expressing 2-4 fold higher cell specific virus titers have been selected. These have been successfully cultured in high cell density, single-use bioreactors up to 40 million cells per mL. Following infection with Sabin poliovirus vaccine strains, a single chromatographic step using a novel membrane has resulted in 90% recoveries at 95% purity.

For IPV, these yield intensifications mean that the entire commercial scale process can be operated in isolators in a footprint of ±30 m^2. Combination of four of these manufacturing units in a single "micro-facility" would be capable of delivering >40 million doses of trivalent sIPV per year.

Performance of the manufacturing process in isolators also allows the manufacture of viral vaccines that currently require high biological safety containment in an inexpensive facility design. This presentation will discuss the technologies used in the vaccine manufacturing platform and data obtained to date on Sabin IPV in more detail.

PROTEOMIC CHARACTERIZATION OF INFLUENZA H1N1 GAG VIRUS-LIKE PARTICLES AND EXTRACELLULAR VESICLES PRODUCED IN HEK-293SF

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Key Words: virus-like particles, influenza vaccine, nLC-MS/MS, extracellular vesicles, HEK-293SF cells

One of the major concerns associated with the use of influenza virus-like particles (VLPs) produced in cell culture as vaccine candidates is their heterogeneous composition. Enveloped VLPs take up the host cell membrane at the budding site carrying out with them not only the viral antigenic proteins but also host cell proteins. In addition, the intrinsic nature of the cells to produce membrane derived vesicles which have similar size to the VLPs and can also contain the antigenic proteins, makes the VLP purification process challenging. Certainly, the expression system and the viral recombinant proteins employed will determine the nature of the proteins within the VLPs. To further characterize cell culture produced-influenza VLPs and contribute to enable their approval as vaccine candidates, the composition and biogenesis of VLPs need to be better understood. In this study we have characterized, by nanoscale liquid chromatography tandem mass spectrometry (n-LC-MS/MS), influenza H1N1 Gag-VLPs produced in human embryonic kidney cells adapted to serum-free medium (HEK-293SF). The cells stably express HA and NA, and the VLPs production occurs following transient transfection with a plasmid containing the gag gene of HIV-1 fused to GFP. Extracellular vesicles (EVs) produced by the unmodified HEK-293SF were also characterized by n-LC-MS/MS. A total of 73 host cell proteins were identified in the VLPs, whereas 98 were detected in the extracellular vesicles. From that, 32 host cell proteins were unique to VLPs while 41 proteins were found in both. Importantly, nucleolin was the most abundant host cell differential protein identified in VLPs while lactotransferrin and heat shock protein 90 were the most present in EVs. This study provides a detailed proteomic description of the VLPs and EVs produced in HEK-293SF as well as a critical discussion of the function of each protein incorporated in both nanoparticles species. The outcome of this research also sheds light on unique target proteins differentially identified either in VLPs and EVs that could potentially be exploited for the development of novel purification protocols to separate EVs from VLPs.

IMPLEMENTATION OF A PROCESS-SCALE ADENOVIRUS PURIFICATION WITH A SINGLE-USE PLATFORM

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Key words: adenovirus purification, single-use platform, clarification, UF/DF, membrane chromatography

Adenovirus vectors are finding increasing application within the vaccine and gene therapy industries. Companies developing adenovirus-based biopharmaceuticals will benefit from single-use process platforms that are quick and easy to install and have been demonstrated previously to produce purified adenovirus. The platform can be adapted to new adenovirus products with a minimum investment of time and resource in process development activities. We have assembled a platform from existing and proven technologies, available from lab to production scale, that meet the quality requirements of the vaccine industry. The platforms includes clarification, ultrafiltration/diafiltration, chromatography and sterile filtration steps. The approach has been successfully demonstrated for the purification of an Adenovirus serotype 5 vector, at the 20 L scale. It allows the complete purification of the vaccine in one day.

MEMBRANE CHROMATOGRAPHY CASSETTES FOR BIND & ELUTE APPLICATIONS OF VIRUSES AND LARGE PROTEINS

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Key words: Virus purification, membrane chromatography, large scale, modularity, scalability

For flow-through polishing applications, membrane adsorbers have become a well-established technology. However, there is an increasing demand for bind and elute purifications for larger targets as adeno- and lentiviruses, virus like particles (VLP) and influenza. The reason is the higher binding capacity of macroporous membranes compared to conventional resins having much smaller pores and excluding them by size. But capture applications with such devices suffered from the current size limitation of 5 liters. Here we describe a modular cassette system which has been tested for scale-up and flow performance in comparison with void volume optimized capsules. The goals were to create a system up to 20 L membrane volume which can be optionally expanded to ~100 liter and, be able adapt exactly to the size needed (modular), using the same 4 and 8 mm bed height as the capsules and membranes for single- or intra batch re-use.

INFLUENZA VIRUS CAPTURE USING MEMBRANE CHROMATOGRAPHY: IMPROVING SELECTIVITY BY MATRIX DESIGN AND PSEUDO-AFFINITY LIGAND INTERACTIONS

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Key words: Influenza purification, membrane chromatography, sulfated cellulose, binding capacity, recovery
Because next generation cell-based influenza vaccines have to be produced faster and in greater quantities than traditional vaccines, future purification processes will require more efficient unit operations for their isolation and purification. Membrane chromatography has already demonstrated a number of positive characteristics for the bind&elute purification of viral particles like e.g. adenoviruses or influenza viruses. The technology not only addresses the diffusion limitations of porous particle media but also offers dramatic advantages in binding capacity in a disposable format. Therefore, the last remaining challenge for the easy adoption of this technology in the vaccine industry represents selectivity and recovery. We present here a novel cellulose based stationary phase whose active specific surface area is designed for maximum virus accessibility. The resulting gain in selectivity and recovery but also in binding capacity is further maximized by using highly selective pseudo affinity ligands for influenza viruses.

DEFINING THE MULTIPLICITY AND TYPE OF INFECTION FOR THE PRODUCTION OF ZAIRE EBOLA VIRUS-LIKE PARTICLES IN THE INSECT CELL BACULOVIRUS EXPRESSION SYSTEM

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Key Words: Ebola virus, ZEBOV-VLP's, coinfection, hemorrhagic fever, immunogenic response.

Ebola virus hemorrhagic fever affects thousands of people worldwide with high mortality rates. The Ebola virus has a short incubation time between 2-21 days and death usually occurs within 4-10 days[1]. Ebola virus disease is characterized by a sudden onset of fever, weakness, headache, diarrhea and vomiting, internal and external bleeding[2]. In the Filovirus family, Zaire Ebola virus (ZEBOV) is the most aggressive and virulent species, its fatality rates have been reported to be up to 90%[3]. Even when important advances in vaccine development have occurred, the need of safe and effective vaccines persists[4]. An alternative is the production of virus-like particles, which are formed by the recombinant virus structural proteins that self-assemble into highly immunogenic structures[5]. The ZEBOV contains three main structural proteins: the glycoprotein (GP), the viral matrix protein 40 (VP40) and the nucleoprotein (NP). GP induces humoral and cellular responses by itself but when VP40 is co-expressed, the immune response increases in a mouse model[6]. NP determines the structure of the resulting VLP. To our knowledge, there is no information about the production conditions that result in coexpression and assembly of ZEBOV recombinant proteins. In this work, a multifactorial experimental design was used to evaluate 32 different conditions for the production of the ZEBOV structural proteins utilizing the insect cell-baculovirus expression system technology (BEST). Multiplicity (MOI = 0.1 or 5 ufp/cell) and consecutive times of infection (0 or 6 hours after the first infection) were the principal factors, and the production of each recombinant protein and assembly of VLP were the evaluated responses. We observed that multiplicity of infection had an impact over expression of the recombinant proteins, higher multiplicities increased yield and VLP assembly. In contrast, later times of infection reduced the production of each protein. The initial presence of VP40 resulted in a higher concentration of NP. The conditions where the simultaneous expression of the three structural proteins and where VLP were detected were identified. The highest MOIs for bacVP40 and bacGP were needed. bacNP should be added during the initial infection with an MOI of 0.1, or at 6 hpi at MOI of 5. The obtained ZEBOV-VLPs were similar to native virus. The obtained VLP are a candidate vaccine under evaluation.

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STRUCTURE-BASED VACCINE DESIGN BY ELECTRON MICROSCOPY

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Key Words: (cryo)TEM, 3D protein structure, conformational stability, epitope mapping

Modern vaccine design relies on multiscale, interdisciplinary efforts that take advantage of innovative technologies such as *in silico* identification of antigens, high throughput screening of antigen immunogenicity, and gene expression profiling to predict host immune responses. In recent years, structural analysis has played an increasingly important role in vaccine development as a means to improve antigen stability, immunogenicity and large scale production. Transmission electron microscopy (TEM), and in particular cryo-TEM, is an established and powerful imaging technique applicable to many specimens, including the three-dimensional (3D) reconstruction of macromolecules and their associated complexes to high resolution. The technique is parsimonious in its material requirements and captures the specimens in their fully hydrated state, close to their native environment. The resolution of cryo-TEM reconstructions was limited to the subnanometer range until the recent development of direct electron detectors and improvements in image processing software, which has led to a so-called “resolution revolution” in the cryo-TEM field. Several protein structures have now been solved at near atomic resolution, establishing the technique as a viable alternative to X-ray analysis for high resolution structure determination. We have determined several structures with and without bound compounds at 2.9 Å – 3.6 Å resolution, which are being integrated into drug discovery and development workflows by our clients. Here we present the 2.4Å resolution structure of apoferritin determined with our Titan Krios electron microscope as an example of the cryo-TEM services available at NIS. These services are significantly enhanced with unique access by NIS to a new instrument, Spotiton, a robotic device that dispenses picoliter-volumes of sample onto a self-blotting nanowire grid as it flies past en route to vitrification. This provides several advantages over standard vitrification methods, including more automated and reproducible preparation of specimens and reducing the deleterious effects of particles interacting with the air-water interface.

While high resolution 3D structure determination by cryo-TEM is at the forefront of structural biology, averages of 2D projection images at moderate resolution in negative stain or vitreous ice can also provide a wealth of information that may be difficult to obtain using other methods. This is illustrated in a number of case studies, including (1) mapping of neutralizing epitopes on the CMV pentameric glycoprotein complex; (2) mapping of neutralizing epitopes on the HIV-1 envelope glycoprotein trimer; (3) assessment of structure and conformational stability of pre- and post-fusion RSV-F protein; (4) characterization of novel adjuvants and protein delivery systems. In summary, both the moderate resolution TEM and high resolution cryo-TEM methods are well suited to extensively characterize antigen structure-function relationships, some of which may be refractory to other experimental methods.

REDUCING RISKS WITH A SERUM-FREE MEDIUM FOR MRC-5 BASED VACCINE PRODUCTION

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Key Words: MRC-5, Diploid, Varicella Zoster Virus, Serum-Free

The World Health Organization has set limits to the amount of bovine serum albumin (BSA) in vaccines to 50ng/dose. Vaccines that are produced with MRC-5 or other diploid cells are cultured in classical medium with bovine serum. Fetal bovine serum contains on average 23g/L BSA which adds a burden to downstream vaccine formulation. To reduce risks associated with bovine sera, we have developed an animal origin-free vaccine production medium for diploid cells. The medium is paired with a serum-free growth medium that supports direct recovery from thaw and adaptation-free expansion, while resulting in performance that is comparable to serum-containing medium. We confirmed virus production with varicella zoster virus and vesicular stomatitis virus (as an analog to rabies virus) and demonstrated titers that were up to one log higher than classical medium control cultures. Taken together, we have developed a workflow for diploid cells consisting of a serum-free medium for growth and an animal origin-free medium for virus production. By switching to a serum-free process, vaccine manufacturers can reduce dependency on serum, production and purification costs, and increase product consistency and safety.

PROCESS ECONOMICAL EFFECTS OF IMPLEMENTATION OF READY-TO-USE MICRO CARRIERS IN CELL-BASED VIRUS VACCINE PRODUCTION

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Key Words: Microcarrier, cell, adherent, Cytodex™ 1, ready-to-use.

Micro-carriers are used as support for the growth of adherent cells. By providing a large cultivation surface in bioreactor cultures, micro-carriers have replaced, to a great extent, cultivation in Cell Factory™ systems or roller bottles over the last decades.

At Sanofi Pasteur, one of the world leaders in human vaccines, Cytodex™ 1 microcarriers have been used in the production of viral vaccines on Vero cells for several years. In accordance with the supplier's recommendation, the microcarriers that are delivered dry are swollen in buffer, washed, and heat-sterilized before use. Since October 2016 a ready-to-use Cytodex™ 1 alternative, delivered presterilized by gamma irradiation, is available.

Before implementing the change, the presterilized alternative was first evaluated with regards to reduced preparation time and cost. With a two-year shelf-life, the presterilized alternative reduced utility cost and added flexibility to operations by decreasing the need for steam and stainless steel materials in viral production facilities, and in alignment with extended use of single-use bioreactors equipment.

The second step was to compare the cell growth and viral productivity using this ready-to-use alternative with that of the prior referenced product in place. Both cell growth and viral productivity were comparable between the two products, which supported further the documentation for the implementation of this ready-to-use alternative in GMP manufacturing for new R&D vaccine projects. The qualification process covered technical, quality, and analytical aspects based on the supplier documentation, and internal analyses and justification regarding our requirements in upstream vaccine production.

While the presterilized Cytodex™ 1 microcarriers are now implemented in process development for new vaccines and qualified for manufacturing of clinical batches of new vaccine products, the next step will be to evaluate the benefits and impacts of replacing the microcarrier reference product with the gamma sterilized alternative on industrial products.

BIOPROCESS ENGINEERING OF INSECT CELLS FOR ACCELERATING VACCINES DEVELOPMENT

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Key Words: Insect cells, Virus-like particles, Bioprocess intensification, Vaccines.

Insect cells emerged as a powerful and versatile platform for vaccines production, mostly using the lytic baculovirus expression vector system (BEVS). Stable expression in such hosts has been increasingly explored to circumvent BEVS-related drawbacks, but protein titers achieved to date are still seemingly low. The design of new or improved cell factories and bioprocess intensification strategies are therefore necessary to increase productivities and thus accelerate implementation of stable insect cell lines as a fast, cost-effective platform for vaccines manufacturing.

In this work, we implemented an innovative site-specific recombination strategy based on flipase-mediated cassette exchange technology to establish reusable insect (*Sf*-9 and High Five) cell platforms for fast production of enveloped virus-like particles (VLPs). Influenza M1 and HIV Gag proteins were evaluated as scaffolds, and proof-of-concept demonstrated using two membrane proteins: the influenza HA protein (for vaccines) and the human beta-2 adrenergic receptor (for drug screening or antibody discovery).

Aiming to improve production yields in developed stable cell lines, two bioprocess engineering schemes were evaluated (either individually or in combination): (i) adaptive laboratory evolution of insect cells to hypothermic culture conditions, and (ii) supplementation of insect cell cultures with productivity enhancers. The stable cell line expressing HIV Gag-VLPs was used as model. Under hypothermic culture conditions, adapted *Sf*-9 cells expressed up to 30-fold more HIV Gag-VLPs than non-adapted cells. Noteworthy, the element driving such increase in productivity is the adaptation process and not the temperature shift as the latter alone leads to lower production yields. A more modest increase in productivity (up to 7-fold) was observed when supplementing non-adapted cell cultures with productivity enhancers NaBu and DMSO. No synergistic effect was observed when combining adapted cells and supplementation with productivity enhancers. Production of HIV Gag-VLPs was successfully scaled-up to stirred-tank bioreactors.

The adapted cell line was then pseudo-typed with influenza HA protein for production of Gag-HA VLPs, and their performance benchmarked against (i) parental *Sf*-9 cells stably expressing Gag-HA VLPs and (ii) insect cells-BEVS, both cultured under standard temperature conditions (27C). Adapted cells showed increased production of Gag-HA VLPs when compared to parental/stable cells, corroborating previously obtained data, but still lower when compared to insect cells-BEVS. Bioprocess intensification strategies are currently under in-house testing to further improve yields of adapted cells and thus shorten the gap between stable insect cells and IC-BEVS.

Overall, the insect cell platforms and bioprocess engineering strategies herein assembled have the potential to assist and accelerate vaccines development.

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A SCALABLE ADENOVIRUS PRODUCTION PROCESS, FROM CELL CULTURE TO PURIFIED BULK

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Key Words: HEK293, Adenovirus, bioprocess production, Biacore™, chromatography

Adenovirus (AdV) vectors are commonly used in cancer gene therapy trials, evaluated in gene therapy and used as vaccines for various diseases. AdV vectors are well studied and are suitable as vaccine vectors due to their ability to infect different cell types, remain episomal and produce stable high titer material. Manufacturing of safe and efficacious clinical-grade virus relies on a scalable and cost-effective production process. In this study, we have combined experimental work and process economy calculations, from AdV production in cell culture to purified bulk product up to 10L scale. An efficient and scalable process for AdV production was developed by evaluation of each process step. The most studied vector is serotype 5, making this a suitable system for process development of AdV vectors. Human AdV5 expressing the green fluorescent protein (GFP) was used for process development. First, suspension HEK 293 cells adapted to serum-free cell culture medium were optimized for AdV production and evaluated in different single use bioreactor systems. Tween 20 was used for cell lysis as a replacement for the traditionally used Triton X-100 (now on the Authorization list (Annex XIV) of REACH, the regulation on Registration, Evaluation, Authorization and restriction of Chemicals). A residual Tween 20 assay with low detection limit was set-up. Filters and conditions for clarification, concentration and buffer exchange by tangential flow filtration were optimized. Anion exchange based capture step alternatives were compared, including different chromatography resins and membrane formats. Finally, core bead technology was evaluated as an alternative to size exclusion chromatography for the polishing step before the final formulation. Analytical methods for virus titer are challenging and depend on purity and quality of the sample. For total virus titer, qPCR and HPLC methods were used. Furthermore, a method based on surface plasmon resonance (Biacore) was developed for analysis of adenovirus titer. For infectious virus titer, we have used a cell based assay with automatic image analysis. Based on analytical data different downstream process alternatives were compared regarding load capacity, recovery and purity and we propose a robust and scalable process with a favorable process economy.

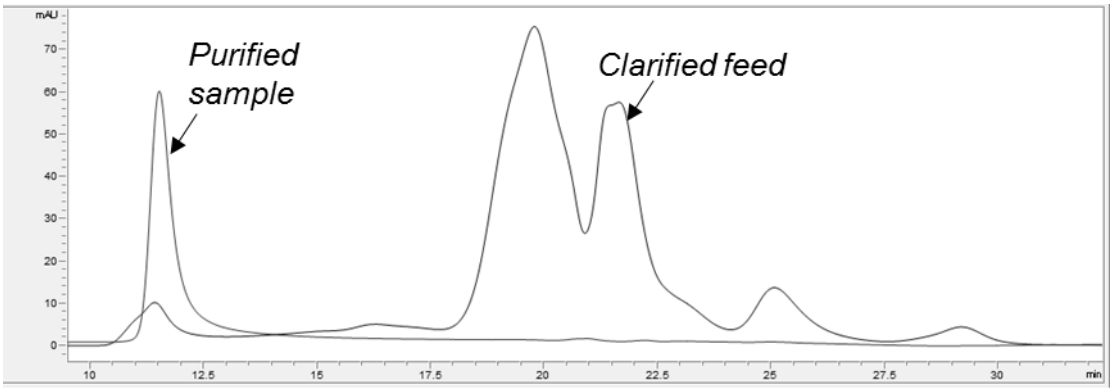

Figure 1 – Size exclusion HPLC analysis of start clarified material and final purified sample from the selected process variant.

VACCINE PROTEIN STABILIZATION IN SILICA

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Key Words: Vaccine, cold-chain, bio-inspired, stabilization, silica

Successful eradication or control of prevailing infectious diseases is linked to vaccine efficacy, stability and distribution. The majority of protein based vaccines are transported at fridge temperatures ('cold-chain') to maintain their potency. However, this has been shown to be problematic. Proteins are inherently susceptible to thermal fluctuations, occurring during transportation, causing them to denature. This leads to ineffective vaccines and an increase in vaccine preventable diseases, especially in low-income countries. Our research utilizes silica to preserve and eventually distribute vaccines at room temperature, thereby decreasing the load on 'cold-chain' logistics. The methodology is based upon sol-gel chemistry where soluble silica is employed to encapsulate, ensilicate, vaccine proteins[1]. This yields protein-loaded silica nanoparticles in the form of a dry powder (figure 1). The material is stored at room temperature and stress tested (heating, 80°C, 2 hours). Subsequently, ensilicated protein is released using a fast chemical process. Silica, silicon dioxide, is an inert biocompatible material with certain ceramic properties that is beneficial in this scenario. The proof-of-concept work was done with a common vaccine antigen: tetanus toxin C fragment[2]. This protein is the immunogenic part of the full tetanus neurotoxin. Analysis of TTCF protein before and after stabilization in silica revealed full retention of protein structure at various levels. Additionally, specific antibody binding indicated retention of immunogenic epitopes (figure 2). These finding suggest that this methodology could reduce or perhaps eliminate vaccine waste. More work will be undertaken to verify protein stabilization and functional retention *in vivo.*

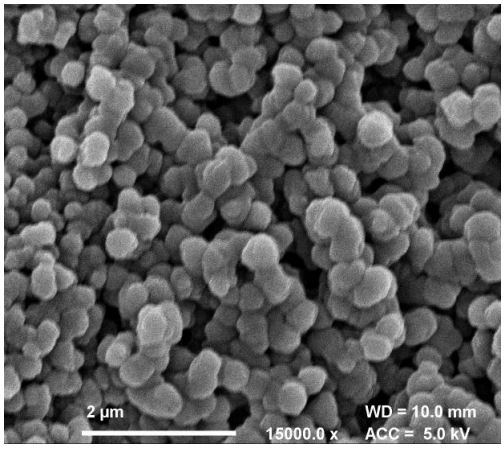

Figure 1. FE-SEM TTCF silica nanoparticles.

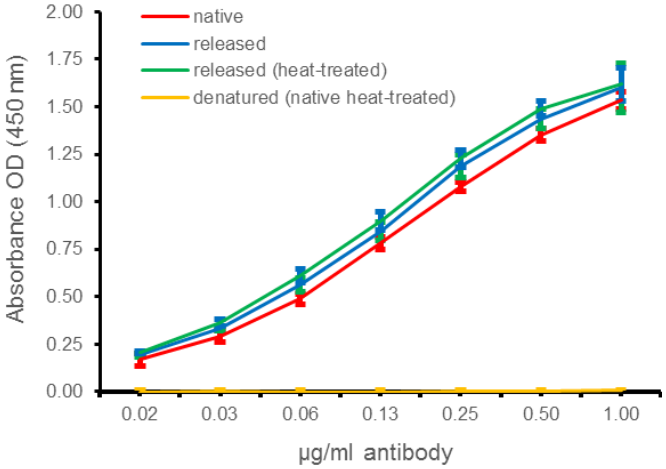

Figure 2. ELISA TTCF of native vs released[1]. TTCF protein samples coated on a high binding, 96-wells, plate were detected using a TTCF specific monoclonal antibody.

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EXPERIMENTAL AND COMPUTATIONAL FLUID DYNAMICS STUDIES OF ADHERENT CELLS ON MICROCARRIERS IN AN AMBR® 250 BIOREACTOR

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Key words: high-throughput process development, microcarrier-based cell culture, fluid dynamics, Vero cells

Interest for microcarrier-based processes for the large-scale culture of adherent cells has recently grow, due to possible application in vaccine and cell therapy. This opportunity drives the need for effective, high-throughput, single-use, process development tools that can be translated successfully into industrial-scale systems. The automated ambr® 250 platform is one such technology, operating at a volume between 100 – 250mL, both high-throughput and single-use. The ambr250 has demonstrated significant success for suspension-based mammalian cell culture applications. However, additional investigations need to be performed on microcarrier-based processes for the culture of adherent cells. The fluid dynamics characteristics of the bioreactor must be sufficiently well understood to enable successful scale-up to larger scale bioreactors. Physical parameters such as fluid velocity, power number and shear stress are important for any cell culture. With microcarriers, there is an additional challenge as the fluid dynamics must take into account the presence of the particulate solid phase. A critical aspect for cell cultivation on microcarriers is the minimum agitator speed required to achieve complete microcarrier suspension, NJS. Under these conditions, the surface area of the attached cells is available for transfer of nutrients (including oxygen) to the cells and metabolites from them, whilst higher speeds hardly increase these transport processes and may lead to damaging fluid dynamic stresses being generated. It is also extremely beneficial to predict the flow dynamics of the stirred tank based on computational fluid dynamics (CFD). Once validated, CFD modelling is a very useful tool for analysing flow patterns, mixing time, mean and local specific energy dissipation rates, shear stress, and other parameters important for scale up in order to optimise the overall bioreactor geometry. In addition to the above fluid dynamic aspects, cell culture studies was also performed in parallel to analyse the cell growth at and around the minimum speed for microcarrier suspension, NJS. The CFD and experimental results with the single-use ambr250 bioreactor will be discussed in detail during the final presentation.

FROM BENCH SCALE TO PILOT PLANT OPERATION: BUSINESS MODELS AND CHALLENGES IN THE BIOPHARMACEUTICAL INDUSTRY IN BRAZIL

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Key Words: Pilot plant, Challenges, Biopharmaceutical industry

Brazil has several challenges to strengthen the biopharmaceutical industry, one of them particularly regarding the ability to translate scientific research into products. Many scholars have been working on this issue and the causalities involved on it in order to contribute to a better understanding of interrelationship between micro and macroeconomic dynamics. Most of the studies are directed to private organizations based in developed countries and aimed to understand the factors of success. Despite the wealth of these approaches, there are few studies about the challenges involved in the transitions from preclinical to clinical stage in the process of Research and Development in developing countries. There are gaps in the understanding of these steps, especially with respect to the requirement to move from the bench to the pilot scale. The main objective of a pilot plant in the biopharmaceutical industry is to demonstrate, with scientific and technological data, the feasibility of a candidate product being transformed into a pilot batch in a GMP certified area. For that, it is necessary to obtain data on yield, reproducibility, repeatability of the established processes, physicochemical stability and thermal stability of the candidate antigen or molecule, formulation parameters and process scheduling. In view of this fact, the present work addresses the issue of industrial pilot plants areas in developing countries from the application of business models approach. The literature about business models provide a practical reference able to assist other organizations to outline the value proposition and to analyze the critical factors for success implementation of pilot plants. This work aims to contribute to the discussion of this topic in Brazil due to the ongoing initiatives for the implementation of pilot plants and to serve as subsidies for public policy. Considering the scenario of the biopharmaceutical industries, where the dynamics of biotechnological and economic transformations has been shown to be complex for the evolution of the product development cycle, Brazil needs not only to improve its infrastructure for Research and Development but also to design and implement appropriate business models for the components of this infrastructure.

IMMUNIZATION WITH FC-BASED RECOMBINANT EPSTEIN-BARR VIRUS GP350 ELICITS POTENT NEUTRALIZING HUMORAL IMMUNE RESPONSE IN A BALB/C MICE MODEL

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Key Words: Epstein-Barr virus, envelope protein, gp350, Fc-based vaccine, neutralizing antibody.

Epstein-Barr virus (EBV) was the first human virus proved to be closely associated with tumor development, such as lymphoma, nasopharyngeal carcinoma (NPC) and EBV-associated gastric carcinoma. Despite many efforts to develop prophylactic vaccines against EBV infection and diseases, no candidates have succeeded in effectively blocking EBV infection in clinical trials. Previous investigations showed that EBV gp350 plays a pivotal role in the infection of B lymphocytes. Nevertheless, using monomeric gp350 proteins as antigens has not been effective in preventing infection. Multimeric forms of the antigen are more potently immunogenic than monomers, however the multimerization elements used in previous constructs are not approved for human clinical trials. To prepare a much-needed EBV prophylactic vaccine that is potent, safe and applicable, we constructed an Fc-based form of gp350 to serve as a dimeric antigen. Here we show that the Fc-based gp350 antigen exhibits dramatically enhanced immunogenicity compared to wild-type gp350 protein. The complete or partial gp350 ectodomain was fused with the mouse IgG2a Fc domain. Fusion with the Fc domain did not impair gp350 folding, binding to a conformation-dependent neutralizing antibody and binding to its receptor by ELISA and SPR. Specific antibody titers against gp350 were notably enhanced by immunization with gp350-Fc dimers compared to gp350 monomers. Furthermore, immunization with gp350-Fc fusion proteins elicited potent neutralizing antibodies against EBV. Our data strongly suggest that an EBV gp350 vaccine based on Fc fusion proteins may be an efficient candidate to prevent EBV infection in clinical applications.

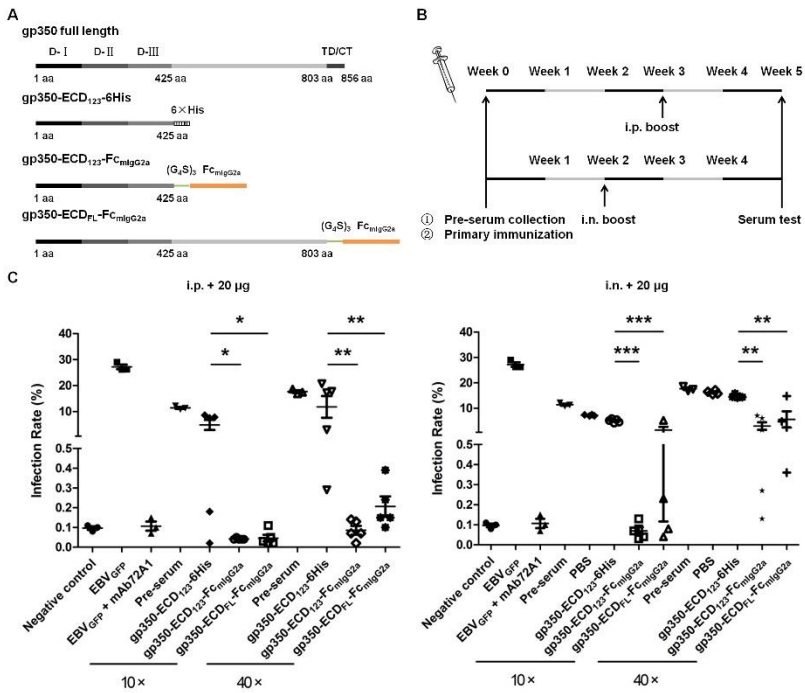

Figure 1. Immunization with Fc fusion antigens potently induced production of neutralizing antibodies. (A) Diagrams for design of Fc-based recombinant gp350 proteins. (B) Diagram of the immunization protocols. Mice were boosted on week 2 for i.n. group or week 3 for i.p. group. (C) Neutralization of EBV_{GFP} infection by sera collected at week 5 post-immunization. Recombinant EBV_{GFP} was preincubated with 10× or 40× dilutions of sera from mice immunized with the indicated antigens. Virus was added to Akata cells and GFP fluorescence was recorded as a measure of infection.

STRATEGIES TO OVERCOME THE AGE-OLD PROBLEM OF IMMUNOSENESCENCE

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Immunosenescence, which is the deterioration of the immune system that comes with age, hampers the development of protective immunity to vaccine antigens. The molecular underpinnings of this process remain poorly understood. Given that systems immunology approaches have been successfully applied towards identifying important molecular features driving productive innate and adaptive immune responses to different vaccines, our goal was to use these approaches to understand, predict, and eventually overcome immunosenescence. To this end, we coupled these systems immunology approaches with highly sensitive in vitro human immune assays to gain insights on specific gene targets that could be modulated to restore the age-dependent decline of immune function.

DEVELOPMENT AND VALIDATION OF A PROPRIETARY MEDIUM FORMULATION FOR RECOMBINANT SUBUNIT VACCINES BY THE BACULOVIRUS EXPRESSION VECTOR SYSTEM (BEVS)

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Key Words: Medium culture, baculovirus system, proprietary formula, medium supplier, animal origin free.

Insect cells & baculovirus expression vector system (BEVS) is an efficient platform for the production of baculovirus vectors and the expression of recombinant proteins or VLPs (Virus Like Particles). This technology is increasingly used in large-scale manufacturing of human and veterinary vaccines. The production of safe and cost-effective vaccines requires the development of animal origin free and low-cost culture media supporting strong insect cell growth, high yield of baculovirus and sustained expression of recombinant proteins or VLPs. In this context, Boehringer-Ingelheim decided to develop its own animal origin free media (with similar or better performance than the commercial one) in collaboration with NRC and suppliers. The different steps, feedback and results will be presented in this poster.

DEVELOPMENT OF AN ANIMAL-COMPONENT FREE INSECT MEDIUM FOR THE BACULOVIRUS EXPRESSION VECTOR SYSTEM (BEVS)

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Key Words: Insect cell, baculovirus expression vector system (BEVS), cell culture medium, media development

Insect cells derived from *Spodoptera frugiperda* have been widely used with the baculovirus expression vector system (BEVS) for the production of recombinant proteins and adeno-associated viruses (AAVs) due to their ease of culture, scalability in high cell density suspension cultures, and high protein expression levels. Traditionally, insect cells are cultured in an undefined medium containing yeast hydrolysate and cod liver oil, however, there is an increasing push to use chemically defined, animal-component free medium to minimize any potential contaminants and decrease lot-to-lot variability while maintaining high cell growth and production. In this case study, an animal-component free insect medium was developed utilizing Rational Culture Media Design™ and evaluated with Sf9 cells. Using a traditional formulation as a starting point, the final medium was developed by optimizing multiple nutrient groups in the basal medium, replacing the animal-derived components, and screening several yeast hydrolysate sources. By utilizing multifactor design of experiment software, various nutrient groups were screened including amino acids, vitamins, and metals. The metals group was identified to have the most impact on cell growth and productivity, and therefore concentrations of metal components were further optimized. In addition, the animal-derived components in the starting formulation, cod liver oil and cholesterol, were replaced with animal-component free fatty acids and synthetic cholesterol, respectively. The concentrations of these components were optimized to achieve better growth performance and production while also sustaining formulation stability and streamlining manufacturing processes. Finally, yeast hydrolysate is a well-known, undefined component that is crucial for insect cell growth and productivity. To minimize lot-to-lot variability, the yeast hydrolysate concentration was significantly lowered, and multiple yeast hydrolysate sources and lots were evaluated to determine the highest quality source. As a result, an animal-component free insect medium was developed that had improved growth performance and comparable productivity to a widely used commercially available animal-derived medium.

HOW TO LIMIT THE USE OF SERUM IN VIRAL PROCESSES: A GIBCO PERSPECTIVE

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Key Words: serum, vaccine manufacturing, , BHK-21, MDCK, Vero

Vaccine manufacturing targeting animal and human prophylaxis has been relying heavily on the use of sera to reach adequate titers in mammalian cell culture processes. Safety concerns, lack of process robustness, costs of qualification, supply and storage are some of the main challenges faced while using sera. Taking into account these drawbacks, sera has remained one of the principal raw materials in vaccine manufacturing, but with limited supply worldwide and increased demands, notably from the cell therapy industry, sera's poor economic predictability might become a major issue on cost of good models required for vaccine viability. Using Gibco's expertise in designing and manufacturing cell culture media for the past 55 years, we identified 4 approaches to limit the use of serum in viral processes. The first strategy to require limited process rework is by reducing the amount of serum in vaccine manufacturing through use of enriched basal media and/or bovine serum albumin as a substitute. The second option is to identify which step of the manufacturing process actually requires serum supplementation. Indeed, while cell growth in adherent conditions may require sera for expansion, the production phase after infection can sometimes eliminate use of FBS or BSA supplementation, thus simplifying processes and compliance to regulatory guidelines. The third approach, providing virus production is not adherence-dependent, is to adapt the adherent cell line to suspension culture, hence removing the need to provide attachment factors present in sera. Finally, when available, a completely controlled process can be developed using cells adapted to CD media (chemically-defined and protein-free). The Gibco perspective on vaccine technology is to streamline workflows while providing media capabilities to support vaccine manufacturers at large scale. This work will demonstrate that implementing a well-designed strategy to reduce or remove sera from current processes is an effective way to achieve a more robust and scalable viral process.

A PRE-FUSION, TRIMERIC SUBUNIT INFLUENZA HA-BASED VACCINE ELICITS CROSS-PROTECTION BETWEEN HIGHLY DIVERGENT INFLUENZA A VIRUSES

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Key Words: influenza, vaccine

Despite our best efforts to vaccinate against influenza viruses they remain a major cause of morbidity and mortality worldwide, resulting in 3-5 million severe infections and more than 250,000 deaths annually. Constant antigenic changes in circulating viruses means current vaccines must be updated and re-administered annually. This approach is time-consuming and expensive, and is often hindered by mismatches between circulating and vaccine strains. Strain mismatch can contribute to insufficient vaccine efficacy, which has ranged from just 10-60% over the last decade. Furthermore, recent sporadic zoonotic outbreaks of novel highly pathogenic viruses from avian species, to which current vaccines provide no immunity, have been observed, with fatality rates around 40%. This raises serious concerns of a global pandemic with the potential to spread rapidly before a vaccine can be manufactured. Novel approaches to influenza vaccination are clearly needed in order to overcome these limitations with “universal” flu vaccines being the holy grail. We have stabilized recombinant influenza haemagglutinin (rHA) in its native, pre-fusion conformation by the addition of a novel “clamp” stabilization motif to enhance subunit vaccine potency and breadth of protection. Immunisation of mice with clamp-stabilized prefusion rHA elicited a potent neutralizing antibody response (~4-fold improvement over current vaccines). Most importantly, antibodies elicited upon immunisation with clamp-stabilised prefusion rHA showed an 80-fold increase in cross-reactivity to rHA derived from a divergent, highly pathogenic avian virus (H5N1) when compared to the current influenza vaccines. We have also shown that vaccination with clamp-stabilisted rHA based on the H3 subtype (group 2) is capable of providing cross-protection to a challenge with a highly-divergent group 1 virus (H1N1). Ultimately, this approach could represent a potential universal influenza vaccine, providing enhanced cross-protection against both group 1 and 2 seasonal influenza virus strains while simultaneously providing an increased cross-reactive humoral immune response to potential zoonotic pandemic strains.

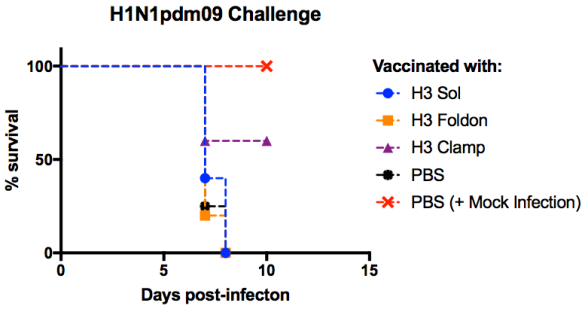

Figure 2 – Mice were vaccinated with rHA with (H3 Clamp) or without (H3 Sol) the molecular clamp trimerisation domain. A previously published influenza vaccine candidate utilizing the foldon trimerisation domain (H3 Foldon) was included as a direct comparison. Mice were challenged with a lethal dose of highly divergent H1N1pdm09 virus. Partial cross-protection was seen only in H3 clamp-vaccinated mice, but not with H3 Sol or H3 Foldon groups.

SUPERINFECTION ARISING IN STABLE LENTIVIRAL VECTOR PRODUCER CELL LINES BEARING COCAL-G ENVELOPE PROTEINS

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Key Words: Lentiviral Vectors, Superinfection, Gene Therapy, Cocal-G, VSV-G

Lentiviral vectors (LV) have been shown to successfully transfer therapeutic genes into dividing and non-dividing cells in laboratory and clinical environments for the benefits of cell and gene therapies. Current LV production chiefly relies on a transient transfection method, wherein HEK 293T cells are transfected with 3-4 plasmids. Such methods have shown batch to batch variability, and increased costs due to the requirements of considerable quantities of cGMP plasmids at clinical stages [1]. This can be circumvented using stable producer cell lines, such as the WinPac cell line, that stably harbor all constructs required for vector production and reliably output LV vectors over long periods of time [2]. However, the commonly pseudotyped LV envelope protein, VSV-G, has difficulty in long term expression and is inactivated by complement [3] and therefore alternatives must be sought. Such alternatives can be found in the Cocal-G envelope protein, which can be expressed long term, is resistant to complement, and bears similarity to VSV-G whereby both derive within the same vesiculovirus genus [4].

A stable LV producer using Cocal-G envelope in the WinPac cell line was produced. Results have illustrated that Cocal-G envelope protein expression leads to superinfection of the LV producing cell line, creating long term instability due to accumulation of the GFP transgene as determined by qPCR. Such superinfection can be prevented by the addition of the non-nucleoside reverse transcriptase inhibitor nevirapine to the cell culture media, leading to protection from superinfection in long term culture. The antiviral can subsequently be removed by buffer exchange in Vivaspin 4 ultrafiltration cassettes (100,000 MWCO), regenerating infectious titre of LV and suggests antiviral addition in upstream production does not negatively impact downstream purification. The cocal enveloped producer cell line was therefore robust enough to be scaled up for large scale LV harvesting as indicated from scaling to a Corning HYPERFlask system.

This work increases our understanding of how LV envelope design may impact superinfection and ultimately specific productivity once cell progress further down a development pathway. In identifying the importance of envelope choice and necessary precautions as a result, work can continue to improve stable LV producers, leading to scalable solutions to address demand for vectors in cell and gene therapies.

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DEVELOPMENT OF SUSPENSION ADAPTED VERO CELL CULTURE PROCESS FOR PRODUCTION OF VIRUSES

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Key Words: Vero cell culture, viral vaccine, cell adaptation, Vero cell media.

Vero cells are considered as the most widely accepted continuous cell line by the regulatory authorities (such as WHO) for the manufacture of viral vaccines for human use. The continuous Vero cell line has been commercially used, after propagation on microcarriers, for the production of rabies, polio, enterovirus 71 and hantaan virus vaccines. Vero cell culture technologies are also explored for productions of many more viral vaccines over the last two decades. The growth of Vero cells is anchorage-dependent, and cells need to be dissociated enzymatically or mechanically for the process of subcultivation. This process is labor intensive and complicated in process scale-up. Adaptation of Vero cells to grow in suspension will simplify subcultivation and process scale-up significantly.

Here we report on the adaptation of adherent Vero cells to grow in suspension using a serum-free and animal component-free medium developed in-house. The maximum cell density and cell doubling time of the suspension adapted Vero cells in batch culture grown in the in-house developed medium were similar to or better than what was observed for the adherent Vero cells grown in commercial media. The growth of suspension adapted Vero culture was successfully scaled up to 3 L bioreactor. The Vero cells from various stages (both adherent and adapted) were tested for their authenticity using a Short Tandem Repeat (STR) analysis. The testing result indicates that all Vero cell samples have 100% concordance with the Vero DNA control sample, indicating the suspension adapted cells maintained their genetic stability.

Productions of vesicular stomatitis virus (VSV) and influenza virus in adherent culture and suspension adapted culture were compared, showing the suspension adapted Vero cell retained similar viral productivity. The volumetric productivity of VSV in the suspension culture was even higher, and was further increased by almost 200 times when culture was infected at higher cell density and with medium replacement before the virus infection. In contrast, the VSV production decreased when the adherent culture was infected at higher cell density. Additional process development revealed that the maximum cell density in batch culture was doubled, reaching $6x10^6$ cells/mL, when the culture medium was replaced during the process of batch culture, which indicates potential for further increases in product titer.

ADDRESSING THE CHALLENGES OF INFLUENZA VIRUS-LIKE PARTICLES PURIFICATION

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Key Words: downstream processing; membrane adsorption chromatography; vaccine production; virus-like particle.

Virus-like particles (VLPs) have been widely used in vaccine development over the last decades [1]. In fact, there are already several approved human vaccines against viruses that use recombinant VLPs as antigen, e.g. for hepatitis B virus and human papillomavirus [2]. Vaccination remains the most effective way to prevent infection with influenza viruses. However, their constant antigenic drift requires an annual update of the seasonal vaccine to prevent influenza epidemics [3-4]. To use the full potential of VLPs as vaccines efficient upstream processing as well as downstream processing (DSP) trains need to be established. The latter is of particular importance as it often accounts for the major biomanufacturing costs. Here we describe the establishment of an improved DSP unit train platform, adapted from virus particles to influenza VLPs, using pseudo-affinity sulfated cellulose membrane adsorbers (SCMA) [5]. An initial clarification step prepares the bulk for the subsequent purification steps. SCMA performance was optimized using a design of experiments (DoE) approach. More than 80% of the product was recovered with removal of host cell protein and DNA above 89% and 80%, respectively. This represents a significant improvement in performance compared to the traditional use of ion exchangers commercially available. Using this SCMA platform for influenza virus particles purification we were able to speed up the process by decreasing the number of DSP steps, to improve the scale-up and to reduce costs due to the removal of other chromatographic steps.

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A GENOME-WIDE CRISPR SCREEN TO GENERATE HIGH-YIELD CELL LINES FOR PANDEMIC INFLUENZA VACCINE PRODUCTION

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Key Words: Influenza, Cell-based vaccine production, CRISPR, Genetic engineering

All influenza vaccines currently sold in Canada require one fertilized chicken egg to produce roughly one dose of vaccine. During pandemic influenza outbreaks, the limited availability of eggs stresses the ability of this method to deliver vaccine in a timely manner (1). Unlike eggs, cell lines grow exponentially, resulting in virtually limitless substrate for cultivating influenza vaccines. This ability to rapidly scale production during periods of increased demand is ideal for effectively responding to pandemic influenza outbreaks. While promising, cell-based influenza vaccine production suffers from low volumetric yield (~10-fold lower) compared to egg-based methods (2).

In this study, a genome-wide screen was used to identify gene knockouts that increase influenza yield in the HEK-293SF cell line. Viral replication is dependent upon a myriad of cellular factors, for example an estimated 9.5% of human protein-coding genes affect HIV-1 replication (3). Many of these cellular factors are components of the innate immune system and actively inhibit virus replication. Identifying these factors will allow the generation of cell lines where they have been knocked out, enhancing influenza yield.

To carry out the screen, knockouts were induced with a lentivirus-vectored, pooled CRISPR/Cas9 library. Cells were then infected with Green Fluorescence Protein (GFP)-tagged influenza. Cells with a favorable environment for influenza replication expressed high amounts of GFP, allowing them to be collected using Fluorescence Assisted Cell Sorting. Next Generation Sequencing was then used to determine which knockouts enhanced influenza replication.

The results of the screen will inform the generation of high-yield vaccine production cell lines based on the HEK-293SF parent line, advancing efforts towards cell-based vaccine production methods that are able to effectively address pandemic outbreaks. The results also offer insights into the host determinants of influenza infection within the unique environment of bioreactor culture.

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PROCESS DEVELOPMENT FOR A FLEXIBLE VACCINE VECTOR PLATFORM BASED ON RECOMBINANT LIFE VIRUS

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Key Words: Life virus, Vaccines, Platform technology.

Vaccines are one of the most important, safe and efficient interventions to protect people from illness, disability and death. In recent years several new viral outbreaks where no vaccines are currently available were reported worldwide. Therefore, the development of flexible processes for the production of vaccines is urgently needed. This project aims at developing a platform process for the production of different viral vaccines. The core technology is based on the fact that large recombinant genes coding for selected, foreign antigens can be inserted into the genome of a well-established virus vaccination vector. The vaccine delivers the selected antigens directly to macrophages and dendritic cells, the most potent and effective antigen-presenting cells, thereby triggering a specific immune response to the selected antigens. As a replicating vector, the vaccine continuously expresses antigens even after immunization. This setup results in a powerful, antigen-focused immune response, which is expected to confer long-term immunity as shown for the measles vaccine.

The challenges in production process design for such a vaccine are the establishment of a robust cell expansion and infection strategy as well the development of efficient downstream processing methods including several chromatography principals, ultra-diafiltration and employment of bio recognition principles. The implementation of a meaningful real-time process monitoring/characterization concept furthermore serves as a basis for reliable in-process control strategies (e.g. the prediction of the optimal infection/harvesting time point).

HER1 THERAPEUTIC CANCER VACCINE: AN ACTIVE IMMUNOTHERAPY TREATMENT FOR PATIENTS WITH TUMORS EXPRESSING THE RECEPTOR OF EPIDERMAL GROWTH FACTOR (EGF-R)

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Key Words: receptor of epidermal growth factor (EGF-R), EGF-R extracellular domain(HER1-ECD), cancer vaccine

Her1 vaccine: It consists of immunizing patients with positive tumors for the epidermal growth factor receptor (EGF-R) with a preparation of EGF-R extracellular domain(HER1-ECD) adjuvanted in VSSP (very small size proteoliposomes) and Montanide ISA51. VSSP adyuvant confers to vaccine the capacity to activate dendritic cells and polarize the immune response towards a TH1 immune pattern, developing TCD8+ cells and antibodies with anti-metastatic effect.

The extracellular domain of the epidermal growth factor receptor was obtained from HEK293 transfectome by using a productive process in a bioreactor in perfusion as mode of fermentation. The protein was purified by immune-affinity chromatography by using specific anti-EGF-R antibody.

Phase I trial, uncontrolled, open and sequential, was conducted in 25 with hormone refractory prostate cancer patients. Five dose levels of HER1-ECD were scaled: 100, 200, 400, 600 and 800 µg in each immunization. The trial showed that the vaccine was safe, not causing severe or very severe related events. The vaccine preparation was shown to be immunogenic. There was a trend towards the survival benefit in some patients.

At the present time another clinical trial is underway in three locations of advanced solid tumors that overexpress the EGF receptor: prostate, colon and head and neck cancer.

COMBINING NOVEL AND TRADITIONAL APPROACHES OF VACCINE DEVELOPMENT TO OVERCOME THE CHALLENGES OF FIRST-IN-HUMAN TRIAL FOR GROUP A STREPTOCOCCUS

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Key Words: Vaccine, Group A Streptococcus, pre-clinical

For nearly 30 years, from 1979 to 2006, clinical trials for a Group A Streptococcus (GAS) vaccines were banned in the U.S after a study suggested there may be an increased risk of acute rheumatic fever (ARF) in vaccine recipients. That study, conducted in 1968, used a crude M protein vaccine to immunize children – following administration, two children developed ARF (Massell BF, 1969). More than a decade since the FDA lifted the ban, safety concerns are still high and new vaccine candidates for Group A Strep face additional scrutiny entering first-in-human trials. This presentation focuses on a case-study for the development of a new vaccine for GAS diseases, specifically the various strategies used to overcome the safety concerns and maximize the chances for successful testing in clinical trials. VaxForm's proprietary vaccine "VaxiStrep," is the result of a combination of novel and traditional approaches in vaccine development. The novel approach entails using a novel recombinant fusion protein as the antigen. While most researchers have focused on combinations of M protein serotypes as the antigen(s) (up to a 26-valent vaccine), VaxForm chose to avoid the inevitable design complexity and stability issues that arise when formulating a multivalent vaccine. VaxiStrep's antigen is a recombinant fusion protein of two streptococcal pyrogenic exotoxins (Spe), SpeA and SpeB, that are widely expressed in GAS strains. In addition to the selection of a safe antigen, VaxForm performed toxicity studies *in vivo* and *in vitro* (human PBMCs) to further demonstrate its safety. An ELISA was developed to show the lack of molecular mimicry between SpeAB and the protein responsible for ARF. SpeAB detoxification was shown by comparing polyclonal T-cell activation of SpeAB vs wild type toxins in human mononuclear cells (PBMCs). Another important part of vaccine design and development is the adjuvant selection. VaxForm decided to explore aluminum-based adjuvants options. Aluminum adjuvants are the most commonly used adjuvants – there is extensive evidence that show their safety, they are approved by the FDA and used in licensed products. Adjuvant dose-response and adsorption studies for VaxiStrep were performed to narrow down the optimal dose and adsorption rate of the antigen. These types of studies followed the more traditional approach of vaccine development, giving the vaccine candidate a better chance for first-in-human approval and clinical trial success. Finally, another aspect that VaxForm selected as a priority was vaccine stabilization. Long term stability studies were performed that demonstrated a shelf-life of over two years when stored refrigerated. Animal studies comparing the potency of a two-year old vaccine lot to a fresh lot showed similar immune response. In addition, titers to the vaccine are long lasting as antibody titers remained at high levels 14 weeks after the booster injection. Conclusions: Bringing a new concept to clinical trials is always a long and challenging task. Learning and understanding the historical perspective when developing a new vaccine can be key in selecting the best approach. In this case, designing a GAS vaccine that would cause the least safety concern was prioritized due to the history of vaccine-induced ARF. Additionally, to avoid delays in clinical trials, robust efficacy and long-term stability of the vaccine was demonstrated in pre-clinical studies. We believe these choices will maximize the chances for VaxiStrep to reach first-in-human trial.

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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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Key Words: Influenza virus, Vaccines, Candidate Vaccine Virus (CVV), Suspension cell culture, Reverse Genetics.

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 Viral Vaccine (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 A/Puerto Rico/8/34 H1N1 strain, and advances in the large-scale transfection of suspension cultured HEK-293 cells. Transfection in shake flasks was performed using 1ug of total plasmid and $1x10^6$ cells/mL. The supernatant was harvested after 48 hpt and used to infect a new shake flasks at $1x10^6$ cells/mL for virus amplification. 3-L bioreactor was inoculated and transfected at $1x10^6$ cells/mL with 1ug of total plasmid and harvested after 48hpt and the virus generated was amplified in shake flask. Quantification by TCID50, SRID, Dot-blot and TRPS were performed as well as characterization by TEM and HA and NA sequencing. Small-scale transfection in shake flasks generated $1.5x10^5$ IVP/mL after 48 hpt and $1x10^7$ IVP/mL after 96 hpi. For large-scale experiment a 3-L controlled stirred tank bioreactor resulted in supernatant (P0) virus titer of $5x10^4$ IVP/mL and $2.8x10^7$ IVP/mL after only one amplification (P1) in HEK-293 suspension cells. We demonstrate the efficent 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. Thus, this innovative approach is better suited to rationally design and mass produce the CVV within timelines dictated by pandemic situations and produce effective responsiveness than previous methodology

IMPLEMENTATION OF A STRATEGY TO PRODUCE A BROADLY NEUTRALIZING MONOCLONAL ANTIBODY AGAINST ZIKA AND DENGUE VIRUSES

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Key Words: Zika, dengue, antibody, CHO cells.

Dengue and Zika viruses are Flavivirus transmitted by a mosquito bite that cause dengue and Zika fever, respectively, with symptoms including fever, rash and headache. The association of Zika virus infection of pregnant women with the development of microcefalia in the fetus lead to the declaration of Zika as a Public Health Emergency of International Concern by the WHO in 2016. The rapid development of methods of detection, quantification and purification of the virus, among others, is necessary for studying the virus at the laboratory and eventually develop a vaccine. Using molecular biology and cell culture techniques, we implemented a strategy to produce a broadly neutralizing monoclonal antibody against dengue and Zika viruses at the lab.

Signal peptides were selected for high level expression of antibodies in CHO cells, according to previous reports (Haryadi et al. 2015). The sequence reported for the human anti-dengue/Zika IgG1 EDE1 C8, including the sequences of the optimized signal peptides for each chain, were cloned into the Freedom™ pCHO 1.0 vector and CHO-S cells were transfected with the resulting construction. Transfection efficiency was low (13%), however, transfected cells were submitted to selection using methotrexate (MTX) and puromycin (PUR) as selection reagents. Two pools of transfected cells were selected using two concentrations of MTX and PUR, and after selection, 100% cells showed expression of the protein of interest, as determined by a parallel control EGFP transfection. Concentration of selection reagents had no effect in productivity in 6-days batch cultures; therefore, the pool of transfected cells growing in the lowest concentration of MTX and PUR was assessed for stability and productivity in presence and absence of selection reagents. Cells growing in medium with and without MTX and PUR showed stable production of the antibody in 10-days cultures, however, differences were found in μ_{max}, X_{max}, and productivity, with the highest values of μ_{max} and X_{max} for the cultures without selection reagents (μ_{max} = 0.04 h^{-1}, X_{max} = 2.68x10^7 cells/mL), in comparison with cultures with selection reagents (μ_{max} = 0.03 h^{-1}, X_{max} = 2.19x10^7 cells/mL). Productivity was higher for cells growing in medium with MTX and PUR (0.159x10^{-6} ug/cell h) than for cells without selection pressure (0.104 pg/cell h), corroborating the importance of maintaining the selection pressure for optimal expression of the protein of interest in this system. The purified antibody recognized the Zika virus and three serotypes of dengue virus, as observed by dot blot, and according to previous reports that demonstrate that the EDE1 C8 antibody recognizes a quaternary epitope conserved in both viruses (Barba-Spaeth et al. 2016). A more exhaustive evaluation of cell pools is necessary to determine the stability of the expression of the antibody for longer periods of time and to optimize its productivity. This antibody will be used for future research and methods development in our lab. Also, the methodology described here could be used as a start point in the production of other therapeutic antibodies and vaccines.

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OPTIMIZATION OF A FLOCCULATION STEP USING A SCALE-DOWN MODEL WITH 3D-PRINTED IMPELLERS AND FOCUSED BEAM REFLECTANCE MEASUREMENT (FBRM) PARTICLE-SIZE MONITORING

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Key Words: flocculation, FBRM, 3D-printing, scale-down

Flocculation is frequently employed in the purification of biopharmaceuticals to improve the efficiency of downstream clarification steps. This operation entails adding an agent to the cell culture broth that causes debris and other impurities to aggregate into larger particles called “flocs”. Due to their size, these “flocs” can be more readily separated from the product stream via centrifugation or filtration.

To develop an efficient flocculation step, it is necessary to identify the minimum amount of flocculating agent that causes particle aggregation to occur. This concentration can be affected by multiple parameters, most notably the mixing environment of the flocculation vessel. Thus, a scale-down model that closely approximates the geometry and mixing characteristics of the commercial-scale vessel with real-time particle-size monitoring would be an ideal tool for flocculation process development.

This poster describes the development and implementation of such a scale-down model. Custom 3D-printed impellers were created that mimic the geometry and mixing characteristics of future commercial scale options and Focused Beam Reflectance Measurement (FBRM) technology was used to track particle size evolution in real-time. By combining these technologies, the model was able to reduce volume requirements 18-fold when compared to off-the-shelf options while quantitatively determining optimal flocculant concentrations under multiple conditions.

This work relates to the themes in this year’s Vaccine Technology conference in two key ways. First, it describes a process development improvement that led to higher throughput experimentation versus traditional methods, enabling faster movement of the vaccine candidate through the development lifecycle. Second, through consideration of the future commercial-scale in the early stages of development, a robust and realistic process was delivered, decreasing time and money wasted on operations that do not translate to the factory floor.

VACCINATION WITH VIRAL VECTORS EXPRESSING NP, M1 AND CHIMERIC HEMAGGLUTININ INDUCES BROAD PROTECTION AGAINST INFLUENZA VIRUS CHALLENGE IN MICE

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Key Words: vaccines, viral vectors, influenza

Seasonal influenza virus infections cause up to half a million deaths each year, the majority of which are older adults. Annual influenza virus vaccination protects against disease, but in the event of a mismatch between the circulating strain and vaccine strain, vaccine effectiveness is severely impacted. Therefore, there is an urgent need for a vaccine that induces broad protection against drifted seasonal and emerging pandemic influenza viruses. One approach in designing such a universal influenza virus vaccine is based on targeting conserved regions of the influenza virus hemagglutinin (HA), the major glycoprotein on the surface of the virus. Using chimeric hemagglutinin constructs (cHA), the immune system can be primed to produce antibody responses against the conserved immunosubdominant stalk region rather than the variable immunodominant head region. Furthermore, replication deficient viral vectors based on Chimpanzee Adenovirus (ChAdOx1) and Modified Vaccinia Ankara (MVA) virus expressing the influenza virus internal antigens, such as the nucleoprotein (NP) and the matrix protein 1 (M1), are capable of inducing strong influenza specific T cell responses in vaccinated individuals. This is another approach towards a broadly cross-protective influenza vaccine given the degree of conservation of NP and M1 across different influenza virus strains. Here, we combine these two platforms to evaluate the efficacy of a viral vector-based group 2 cHA intramuscular vaccination regime in mice to confer protection against influenza virus challenge of matched and mismatched group 2 strains. We show that vectored vaccines expressing both cHA and an NP-M1 fusion protein, in a prime-boost regimen (with different cHAs given at each vaccination), provide enhanced protection against H3N2 and H10N8 virus challenge when compared to vaccination with cHA alone or NP-M1 alone. The vaccine induced antibody responses against divergent HAs, NP, M1, and whole virus correlated with nature of administered vaccine and extent of protection seen across vaccinated groups. Influenza specific T cell responses were also increased in the vectored vaccines expressing both the cHA and the NP-M1 fusion protein. For further characterization, we are interested in looking at an optimal vaccination regimen, the possibility of an additional boost to induce cross-reactive antibodies, and the nature of the induced antibodies. Overall, these results improve our understanding of vaccination platforms capable of harnessing cellular and humoral immunity with the ultimate goal of designing a universal influenza vaccine.

A NEW PORCINE SUSPENSION CELL LINE (PBG.PK-21) PROVIDES EFFICIENT PRODUCTION FOR INFLUENZA AND YELLOW FEVER VACCINE VIRUSES

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Key Words: Porcine kidney cell, influenza A virus (H1N1 and H1N2), yellow fever vaccine, batch and perfusion bioprocesses

Shifting from egg-based influenza and yellow fever vaccine production towards efficient cell culture-based manufacturing is one of the biggest challenges in viral vaccine manufacturing. Innovative bioprocesses, such as high cell density [1] or continuous virus cultivations [2] improving cell-based viral vaccine production have been shown over the past years. However, finding the right high-yield cell substrate is still pending for many important viruses. Ideally, such host cell lines should enable the efficient production of several virus strains to set-up manufacturing platforms.

Here, we report on a novel suspension cell line (PBG.PK-21 (ProBioGen) derived from immortal porcine kidney cells. These cells were first adapted to chemically defined virus production medium CD-U5 (ProBioGen) and agitated suspension culture. Despite its robust growth these cells were not yet suitable for virus manufacture due to a chronic infection with porcine circovirus 1 (PCV1) that is often found in porcine cell lines. The cells were cured from infection after siRNA mediated suppression of the contaminating virus followed by single cell cloning. Clone number 21 proven to be free from PCV1 over multiple passages and distinguished by high peak cell densities and good productivity for other viruses was chosen for further analysis.

Cell growth, cell metabolism and virus production were characterized in shake flasks and bioreactors. Cell concentration up to 8 x 10^6 cells/mL and a doubling time of 33 h were obtained in batch mode in CD-U5 medium. Furthermore, process intensification using either semi-perfusion in shake flasks or hollow fiber-based perfusion with an ATF2 system coupled to a 0.6 L wv stirred tank bioreactor system was evaluated. In this system, cell densities up to 42 x 10^6 cells/mL were achieved with a cell-specific perfusion rate of 0.07 nL/cell/day.

After optimization of influenza A/PR/8/34 (H1N1) virus production, HA titres of 3.3 log(HAU/100µL) with a cell-specific yield of 5200 virions/cells were achieved in bioreactor fed-batch mode. The PBG.PK-21 suspension cell line also shows potential to be used in a modern cell-based swine influenza vaccine production process for the veterinary market. Indeed, similar competitive virus titres (3.4 log(HAU/100µL)) and cell-specific yields were found for the swine influenza A/Bakum/1832/00 (H1N2) virus in batch mode. Finally, tests were also conducted regarding yellow fever virus production (live-attenuated 17DD YFV, RKI). In first scouting experiments, promising titres up to 3 x 10^6 PFU/mL were obtained in batch mode.

First attempts towards process intensification using ATF-based perfusion systems with manually-adapted perfusion rate showed successful maintenance of cell-specific influenza A virus yields at high cell density. Titres up to 4 log(HAU/100µL) were obtained with 42 x 10^6 cells/mL. The glycosylation profile of influenza A/PR8/34 produced in PBG.PK-21 cell line was also analysed and will be compared to profiles of other cell lines.

Overall, PBG.PK-21 suspension cells show a great potential to become the cell substrate of choice for many existing and new viral vaccine processes in next generation high-yield human as well as veterinary vaccine manufacturing.

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HILIC-LC/MS METHOD FOR NON-DERIVATIZED AMINO ACID ANALYSIS IN SPENT MEDIA

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Key Words: Hydrophilic interaction chromatography, mass spectrometry, Multiple Reaction Monitoring, Amino Acid Analysis, Spent Media Analysis.

Spent media analysis is vital to vaccine upstream process development and optimization. Determination of amino acid (AA) concentrations in culture media is essential to understand the changes in cell culture conditions over time and also to design optimal feed strategy to improve production economics. Amino acids are highly polar and most have low UV absorbance. Therefore, derivatization by Ortho Phthalaldehyde (OPA) and Fluorenylmethoxy Chloroformate (FMOC) is necessary to improve retention on reverse phase columns and to enhance sensitivities on UV/florescence detectors. This process is labor intensive and time consuming. In the current approach, we applied hydrophilic interaction chromatography (HILIC) and triple quadrupole mass spectrometry (MS) to quantitate non-derivatized AAs extracted from culture media. Samples are collected from the bioreactor at different time points. Prior to extraction, a mixture of C^{13}/N^{15} labeled AAs is spiked as internal standards (IS) to normalize variabilities in extraction recoveries. AAs and IS are extracted by adding 4 parts of 50% acetonitrile in 20mM Ammonium formate in water (pH=3) to 1 part of culture media sample. The samples are vortexed and centrifuged at 12000 RPM for 5 min to remove the cells and protein precipitates. Supernatants are directly injected on to HILIC–Z column (Agilent Technologies, 2.1X100mm; 2.7uM) connected to SCIEX API 4000 triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. The method uses 20 minute normal phase gradient (buffer system: 20mM Ammonium formate in water and 20mM Ammonium formate in 90% acetonitrile) at a flow rate of 0.5ml/min to resolve 17 amino acids. Data analysis is performed by Analyst software. Ratios of AA and corresponding IS peak areas vs. concentrations are plotted to generate calibration curves for each AA. Our method demonstrated nano-molar detection sensitivities and a linear range of 75nM to 2.5µM for most of the AAs. This method also exhibited excellent resolution between isobaric AAs Leucine and Isoleucine. In the poster presentation, we will present method qualification data in detail. We will also discuss application of this method to two independent vaccine upstream process development studies, one is associated with microbial fermentation process and another involved mammalian cell culture process.

PROCESS DEVELOPMENT OF CHROMATOGRAPHY-BASED PURIFICATION ON PANDEMIC INFLUENZA VIRUS-LIKE PARTICLE BASED VACCINES

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Key Words: Virus-like particles (VLPs), Avian influenza virus

Since 2013, the H7N9 avian influenza virus is considered a threat to global public health. The development of the H7N9 avian influenza vaccine is one of the most effective strategies to prevent influenza pandemics. Virus-like particles (VLPs) influenza vaccines is non-infectious viral structural proteins. Not only to retain the ability to produce neutralizing antibodies against to viral surface protein, but also safer than the conventional inactivated vaccines. In our previous study, we successfully expressed three structural proteins, hemagglutinin (HA), neuraminidase (NA) and matrix (M1) from influenza A / Taiwan / 1/2013 (H7N9) of the VLP in insect cells. In this study, we present a downstream purification method for the VLP platform. The purification process involves microfiltration, chromatography (using ion exchange, affinity and gel filtration combinations), concentration, diafiltration and sterile filtration steps. In this study, 600 ml of the harvest from the baculovirus expression system was used. The characteristics of VLP volume were examined by HA assay, SDS-PAGE and negative staining transmission electron microscopy (TEM). The overall recovery of HA protein was approximately 38%. In the evaluation of immunized mouse, such virus particles have been shown the HI titer >256. This study demonstrated the chromatographic-based purification process can provide an effective VLP vaccine production for the preparation of the H7N9 influenza pandemic.

Δπ=0 REVERSE OSMOSIS ENRICHES A HIGH OSMOTIC PRESSURE SOLUTION FROM A LOW-TITER FERMENTATION BROTH TO A SATURATED SOLUTION OR SALT FORM USING RO AND NF MEMBRANES

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Key Words: Δπ=0 reverse osmosis, reverse osmosis, enrichment, fermentation broth

Diverse biotechnology products are produced by microbial or eukaryotic cell fermentations in aqueous solutions. Removal of water is inevitable to enrich the product into a concentrated solution or into solid forms (such as crystals). The theoretical minimum energy required to remove 1 m3 of water is 716 kWh for thermal methods and 1 kWh for reverse osmosis (RO). In practice, the thermal methods equipped with heat energy recycling needs about 25 kWh to remove 1 m3 of water, and the RO methods needs about 4 kWh since extra energy (3 kWh) is required to operate pumps and other facilities in a plant. In general, membrane processes need less energy than thermal processes since there is no phase change in the separation processes and do not damage heat-sensitive biotechnology products. While both RO and NF membranes are permeable to water, RO membrane retains NaCl molecules and NF membrane is permeable to NaCl molecules, which is useful to remove inorganic salts from the products. Unlike thermal processes, the application of the membrane processes is limited by high osmotic pressure as the product solution is enriched by removing water. Chang et al. (2013) proposed a concept of osmotic pressure-free reverse osmosis (Δπ=0 RO) that overcomes this limitation and allows concentration of any solution with high osmotic pressure to its saturation point and further to crystal form. Δπ=0 RO, a two-component system, is distinct from 3-component forward osmosis and does not require the third component (draw component or extraction solvent) that must be separated from the aqueous solution at the end. This presentation will compare (1) ways of Δπ=0 RO technologies in desalination, and, furthermore (2) dewatering and desalination of high osmotic solutions of NaCl (343 bar), volatile fatty acids (400 – 600 bar), and fuel ethanol (6000 bar) with thermal separation methods in terms of energy consumption and potential of Δπ=0 RO technology.

1. Chang et al. (2017), US patent 14,764,975(2015, 07,30), registration in progress

UPSTREAM PROCESS INTENSIFICATION USING VIRAL SENSITIZER TECHNOLOGY

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Upstream process intensification technologies will play an increasingly important role to cater to growing global demands in vaccines and other viroceuticals including gene therapy vectors and oncolytic viruses. We have discovered and developed viral sensitizer technology, a collection of compounds that improve virus growth with potential utility in a multitude of applications, including manufacturing. We have found that viral sensitizer formulations enhance viral production to varying extents depending on the virus and manufacturing substrate. Productivity can be improved substantially in commonly used production cell lines. In cell culture applications, viral sensitizers can be combined with other process intensification technologies including fixed bed macrocarrier-based bioreactors, as well as commercially available serum-free media and growth supplements. To support commercial implementation, we have developed analytical tools to track compounds during production.

MOLECULAR UNDERSTANDING OF THE SERUM ANTIBODY REPERTOIRES AFTER SEASONAL INFLUENZA VACCINATION AMONG DIFFERENT AGE COHORTS

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Key Words: antibody repertoire, influenza, aging, influenza vaccination, serological repertoire

Numerous influenza vaccination studies based on bulk serology have indicated that the antibody responses to the vaccine markedly decrease in the elderly. However, whether such decline results from the changes in the overall quantity or the quality of the circulating antibodies in serum remains unknown. Utilizing novel antibody repertoire profiling technologies, combining tandem mass spectrometry (LC-MS/MS) and high-throughput sequencing, we investigated the influenza-specific serological repertoires of 10 donors ranging from 26 to 70 years old vaccinated with Fluzone® 2013-2014 and/or 2014-2015. In particular, we determined the serum antibodies that are specific to the H1 or H3 component of the vaccine or cross-reactive between the two (H1+H3) and examined their relative quantitative distributions. Our data indicate that the proportion of H1+H3 antibodies significantly increases in the elderly and that the somatic hypermutation rates of the influenza-specific antibodies are higher in the elderly. These results suggest that the repeated exposure to the different virus subtypes could have led to the prolonged selection of H1+H3 antibodies targeting highly conserved epitopes. To evaluate the potency of the antibodies circulating in different age groups, we recombinantly expressed a number of representative monoclonal antibodies isolated from the donors in different age groups for further characterizations. Overall, our analysis suggests that the influenza-specific repertoire in the elderly may converge toward shared epitopes but the quality of the antibodies can be superior in terms of cross-reactivity. However, because the antibody repertoire “shrinks” as we age while targeting more conserved epitopes across different influenza subtypes, it is possible that the elderly is particularly susceptible to significantly altered strains. Collectively, profiling vaccine induced serological repertoires among different age cohorts can provide unprecedented insights regarding humoral immunity associated with age and a potential explanation for the vulnerability of the elderly.

FORMULATION DEVELOPMENT OF A RECOMBINANT PROTEIN BASED NON-REPLICATING ROTAVIRUS (NRRV) VACCINE CANDIDATE: ANTIGEN-ADJUVANT-PRESERVATIVE INTERACTIONS

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Key Words: Rotavirus, Stability, Adjuvant, Vaccine, Preservative

Rotavirus is the leading cause of acute diarrhea and gastroenteritis among infants and young children worldwide. Over 215,000 children under five years of age die from rotavirus infection each year, mostly in developing world[1]. Currently two live attenuated oral rotavirus vaccines are available globally (Rotarix® and RotaTeq®) to reduce the burden of this disease with an efficacy of >90% in developed countries[2]. Vaccine efficacy is lower, however, in developing countries due to a variety of factors[3]. To this end, a non-replicating rotavirus (NRRV) vaccine candidate, containing three recombinant protein antigens (P2-VP8-P[4], P2-VP8-P[6] and P2-VP8-P[8]), is being developed by PATH and its partners as a trivalent vaccine for use in the developing world[4]. This trivalent rotavirus vaccine candidate includes the three antigens from the most prevalent serotypes associated with >90% of rotavirus gastroenteritis worldwide.

In the present study, the following formulation development issues were examined: (1) establish stability-indicating physicochemical assays for a NRRV protein antigen (P[8]) bound to an aluminum hydroxide adjuvant (Alhydrogel®), which include primary and higher-order structures, chemical and conformational stability of the protein on Alhydrogel, and the ability to desorb the antigen from Alhydrogel; (2) examine the adsorptive capacity and coefficients of Alhydrogel® for the P[8] antigen in several candidate drug product formulations; (3) investigate the effects of binding to Alhydrogel® and the addition of two antimicrobial preservatives (2-phenoxyethanol or thimerosal) on the structural integrity and conformational stability of P[8], the latter of which were found to be potent destabilizers of the antigen; and (4) monitor the real-time and accelerated storage stability over 3 months of P[8] bound to Alhydrogel® in several candidate formulations with and without thimerosal at different temperatures. In the absence of preservative, the P[8] protein antigen was overall stable with only a small amount of Asn deamidation observed in samples stored under real-time (4˚C) or accelerated (25˚C) temperatures. Similarly, little (if any) changes were observed in the real-time stability of the antigen on Alhydrogel® in the presence of thimerosal. Under accelerated storage temperatures (25 or 37˚C) however, the preservative caused an increase in inter-molecular disulfide bonding, decrease of apparent enthalpy as measured by DSC, and a decrease in *in-vitro* antigenicity. Similar stability studies are currently ongoing with the P[4] and P[6] protein antigens.

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DISRUPTIVE MICRO-FACILITY FOR AFFORDABLE VACCINE MANUFACTURING

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Key Words: upstream & downstream process intensification

Univercells is offering disruptive manufacturing platforms based on innovative upstream and downstream approaches, aiming at increasing the availability and affordability of biotherapeutics.

The use of fixed-bed high-density bioreactors with sequential in-line clarification and capture operations creates an intensified and integrated process, resulting in a drastic reduction of footprint and capital expenditure.
By designing a flexible process that can easily be adapted to any viral vaccine without hardware modification, Univercells wishes to reduce the burden of developing adapted manufacturing processes for newly developed vaccines, facilitating their way towards commercialization.

With this process, viral vaccines such as polio can be produced in a miniaturized fully-contained isolator for increased safety, leading to a tremendous impact on the factory design, CAPEX and cost of manufacturing. This isolator-based bioproduction unit can be rapidly deployed locally, maximizing availability of essential biotherapeutics while offering an alternative to transport & storage problematics.

Such micro-facility is able to produce large quantities of vaccines at very low cost, offering a sustainable solution to the next challenges of bioproduction, such as the increasing demand of affordable vaccines for campaign-based immunization, or for the ever-increasing gene therapy segment.

ORBITAL SHAKEN BIOREACTOR FOR INFLUENZA A VIRUS PRODUCTION IN HIGH CELL DENSITY CULTIVATIONS

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Key Words: orbital shaken bioreactor, viral vaccine production, high cell density, perfusion, influenza A virus

The majority of large scale cell culture processes is performed in stirred tank bioreactors (STR) and process development employs scale down models of the same bioreactor type. However, for screening of clones or process conditions at even smaller scale, shake flasks (SF) represent the most widely used model. Occasionally, SF allow for robust processes that cannot be transferred to STR because sensitive cells cannot cope with mechanical stress in STR due to stirring and aeration. Orbital shaken bioreactors (OSB) are a valuable alternative to STR as the transfer from SF to OSB is simplified because the systems rely on the same basic principles for mixing and aeration (e.g., bubble-free surface gassing). In particular, high oxygen transfer rates and short mixing times combined with low shear stress can also be achieved in OSB. These benefits may be even more pronounced in high density culture processes.

In this study, the SB10-X bioreactor (Kühner AG, Switzerland) was evaluated for the production of influenza A virus. Avian AGE1.CR.pIX cells (ProBioGen AG, Germany) were cultivated in the chemically defined medium CD-U3 (Biochrom-Merck, Germany) in 10 L single-use standard bags with 5 L initial working volume (w.v.) and 70 rpm shaking frequency. For perfusion, either an alternating tangential flow system (ATF2, Repligen, 500 kDa cut-off) or a tangential flow filtration unit (TFF, Spectrum Labs, 0.2 µm cut-off) was used (Figure 1). After infection with influenza virus A/PR/8/34 (H1N1) at a MOI of 0.001, the w.v. was increased up to 9 L while perfusion was maintained, similarly to a hybrid perfusion fed-batch strategy previously proposed [1]. In addition, the shaking was increased to 90 rpm during virus production phase.

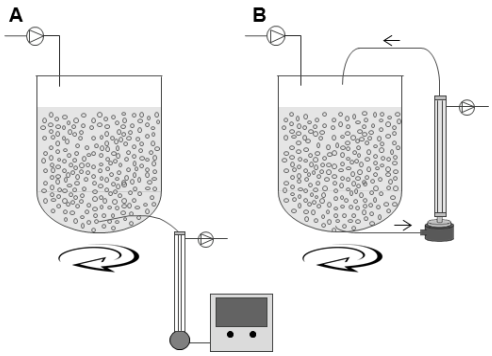

Figure 1. The SB10-X orbital shaken bioreactor was operated in perfusion mode coupled to ATF (A) or TFF (B) with a minimum working volume of 5 L for the 10 L bag. A magnetic drive pump (Levitronix®) was used for recirculation in the TFF.

Both retention devices were successfully coupled to the OSB and concentrations up to 50 x 10^6 cells/mL were obtained, typically with viabilities higher than 90%. We did not observe a decrease of the specific growth rate in the bioreactor system using either perfusion setup. Regarding virus production, we achieved similar or higher HA titers compared to cultivations of AGE1.CR cells in 1 L STR combined with ATF [2]. On average, the cell-specific virus yields (CSVY) obtained in the OSB cultivations were 2.2-fold higher compared to the best ATF cultivations in STR, and a CSVY of 3500 virions/mL was achieved. A comparison between OSB runs in perfusion versus OSB batch revealed a CSVY 2.5-fold higher in perfusion, showing that the typical batch productivity was exceeded by far. On the other hand, infectious virus titers were usually lower in OSB and further experiments are necessary to better understand virus propagation in OSB in case live vaccine production is intended.

To our knowledge, this is the first report on the application of the single-use OSB in perfusion mode and we demonstrate that the technology can be used successfully for virus production.

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VAXARRAY FOR HEMAGGLUTININ AND NEURAMINIDASE POTENCY TESTING OF INFLUENZA VACCINES

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Key Words: Influenza, vaccine, potency, VaxArray, immunogenicity, neuraminidase, hemagglutinin

Practical methods to measure the potency of new influenza vaccines are needed as alternatives for the standard single radial immunodiffusion (SRID) assay. VaxArray assays for influenza hemagglutinin (HA) and neuraminidase (NA) have been developed to address this need. In this report, we evaluate the use of these assays to assess the potency of HA and NA of an A/H3N2 subunit vaccine by determining the correlation between the amounts measured by VaxArray and the immunogenicity in mice. The antibody response after one and two doses of five formulations of the vaccine ranging from 5 µg/mL to 80 µg/mL of HA, was measured by hemagglutination inhibition (HAI) and neuraminidase inhibition (NAI) assays. For hemagglutinin, vaccine potency determined by VaxArray was equivalent to potency measured SRID and these amounts were predictive of immunogenicity, with excellent correlation between potency measured by VaxArray and the HAI geometric mean titers (GMT). Likewise, the amount of NA measured by VaxArray was predictive of the NAI GMT. The VaxArray NA assay reported non-detectable levels of intact NA for a sample that had been heat degraded at 56° C for 20 hours, demonstrating that the assay only measures the native, active form of NA. Similarly, the HA potency measured by VaxArray in this heat-treated sample was very low when a monoclonal antibody was used to detect the amount of antigen bound. Importantly, the force degraded sample induced low HAI titers and the NAI titers were not measurable, supporting the conclusion that the VaxArray HA and NA assays measure the immunogenic forms of these A/H3N2 antigens. The VaxArray platform can therefore be used to assess the potency of HA and NA components of subunit A/H3N2 vaccines.

VAXARRAY POTENCY ASSAY FOR RAPID ASSESSMENT OF "PANDEMIC" FLU VACCINES

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Key Words: Influenza, pandemic, potency, antibody panel, VaxArray

A first-in-class "on-demand" potency assay was developed for flu vaccines produced in response to the most concerning emergent influenza A subtypes. Specifically, the World Health Organization has recommended the development of new candidate vaccine viruses for deadly avian influenza H5 and H7 subtypes. The VaxArray Influenza Pandemic HA (VXI-pHA) potency assay was designed to probe multiple subtype-specific conserved epitopes on the hemagglutinin protein for H5, H7, and H9 subtypes. The goal was to optimize the probability that the ready-to-use assay would work for a new H5, H7, or H9 flu vaccine in order to streamline potency determination, potentially reducing the time to deliver life-saving vaccine by weeks or possibly even months. The performance of this new potency test was evaluated using a large set of influenza viruses and vaccines spanning 16 years of antigenic drift, including the most recent pre-pandemic vaccine being developed against the deadly "5th wave" A/H7N9 virus. Against a panel of 46 potentially pandemic influenza strains, the VXI-pHA assay demonstrated coverage of 93%, 91%, and 100% for H5, H7, and H9 antigens, respectively. The assay demonstrated high sensitivity with linear dynamic ranges more than 150-fold and quantification limits ranging from 1-5 ng/mL. For three production lots of H7N9 monobulk drug substance, the assay exhibited excellent accuracy (100 ± 6%) and analytical precision (CV 6 ± 2%). The high assay sensitivity enabled robust detection and quantification of hemagglutinin in crude in-process samples and low dose adjuvanted vaccines with an accuracy of 100 ± 10%.

APPLICATION OF ANALYTICAL CHARACTERIZATION TOOLS IN PROCESS AND FORMULATION DEVELOPMENT OF LOW COST VACCINES USING THE ULTRA MANUFACTURING PLATFORM

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Key Words: ULTRA, Analytics, Formulation, Multi-dose, Preservatives

In 2016, an estimated 19.5 million infants worldwide did not receive routine life-saving vaccinations according to the World Health Organization (WHO)[1]. Two major limitations in improving global vaccination coverage include the costs associated with vaccine manufacturing and the challenges associated with maintaining a consistent supply. The aim of the 'ULTRA' project (Ultra Low-cost TRansferable Automated Platform for Vaccine Manufacturing) is to standardize the development and production of new protein subunit vaccine candidates at globally affordable costs by creating a generic, low-cost, integrated, and automated vaccine manufacturing platform. In a collaborative effort between MIT, UCL, and KU (*Figure 1*), state-of-the-art analytical tools will be utilized to provide well-characterized vaccine bulk and drug product facilitating process changes and reduced QC costs. Additionally, to ensure the stability and potency of vaccines during production, storage, transport and administration to people in the developing world, vaccines will be formulated with stabilizers/adjuvants/preservatives in a multi-dose presentation to further assist in the development of low-cost vaccines. The initial candidate for the ULTRA platform is a subunit vaccine against rotavirus containing three recombinant non-replicating rotavirus (NRRV) protein antigens, P2-VP8-P[4], P2-VP8-P[6], and P2-VP8-P[8]. These protein antigens are currently being developed clinically by PATH with antigens expressed in *E. coli* [2]. Under ULTRA, these NRRV antigens are being produced in *Pichia pastoris*. Primary structure and post-translational modification analysis of the first generation *Pichia* NRRV strains using SDS-PAGE and LC-MS approaches (intact mass and peptide mapping) revealed the presence of intact protein antigen along with certain levels of N-terminal truncation variants, N-linked glycosylation, and protein aggregation. These analytical observations have guided a 'quality-by-design' approach to introduce site-specific modifications in NRRV antigens to minimize N-terminal truncations, glycosylation and reduce aggregation. Further, given the known compatibility issues of NRRV antigens with preservatives and limited binding to aluminum adjuvants (*see our collaborative poster with PATH*), a 'fast-to-formulate (F2F)' direction has been undertaken to engineer variants of NRRV antigens with potentially improved solubility and stability, especially in the presence of preservatives required for multi-dose formulations. High throughput analytical assays utilizing small amounts of material (~0.5 mg) to provide maximal information on antigen structure, relative solubility, and antibody binding have been established to assess developability of F2F NRRV variants for formulation development.

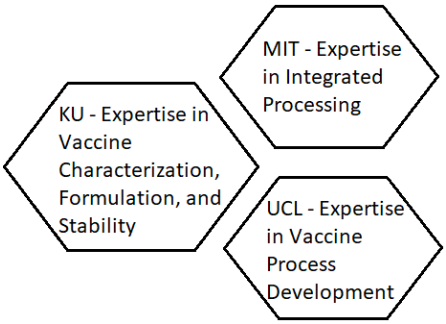

Figure 3 – The academic team of The ULTRA Collaborative

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COMPARAISON OF RABIES VIRUS PURIFICATION USING DIFFERENT METHODS

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Key Words: Rabies virus, vaccine, purification.

Rabies is a viral zoonosis caused by negative-stranded RNA viruses of the *Lyssavirus* genus. It can affect all mammals including humans. Dogs are the main source of human rabies deaths, contributing up to 99% of all rabies transmissions to humans. Infection causes tens of thousands of deaths every year, mainly in Asia and Africa. Most of these victims are children under the age of 15. Vaccination against rabies is still the sole way to fight against the disease.

The aim of this work is to compare the purity of rabies vaccine purified by zonal centrifugation and chromatographic methods, in terms of residual DNA level, host cell protein (HCP) level and the overall recovery yield.
For this purpose, Vero cells were grown under animal component free conditions, on Cytodex 1 microcarriers in VP-SFM medium. Vero cell growth and virus production were previously optimized; studies were conducted in a 7-L bioreactor. Virus replication phase was conducted using perfusion culture mode, viral harvests obtained through the culture were clarified, inactivated by BPL (Beta-propiolactone) and then pooled.

The pooled harvests were purified by zonal centrifugation on a sucrose density gradient. The fractions of interest (11 in total) were pooled and checked for their antigenic activity according to the NIH potency test. They showed an activity of 61 UI/ml. The yield obtained was around 60%.

To improve the overall yield, we have tested during a previous work several chromatography matrices (Sephacryl S200, Sephacryl S300, Sepharose 4FF,...). However the yield obtained was not high, around 40% in the best case. To improve this performance, we tested the Capto Core 700 (GE Healthcare Life Sciences) which a matrice that had a dual-functionality: size separation and binding chromatography, and was specially designed for the purification of large biological products such as viruses. The use of this matrice to purify rabies virus resulted in a yield of 84.5%, which was 2-fold and 1.5 fold higher than that obtained using chromatographic columns or zonal centrifugation, respectively.
We also tested Monolytic chromatographic (CIMmultus™ QA-8 Advanced Composite Column) from BIA Separation. Such kind of media represents a new generation of chromatographic matrices with efficient mass transfer and better hydrodynamic properties. This allows fast and efficient separation of large molecules such as DNA and viruses. In our case, we were able to increase the purification yield to values close to 94%. It was the highest yield obtained compared to other methods used.

Currently the content of the purified fractions collected using the different methods is analyzed to estimate the efficiency of DNA and HCP removal.

COMPARATIVE TRANSCRIPTOME ANALYSIS OF A TRICHOPLUSIA NI CELL LINE REVEALS DISTINCT HOST RESPONSES TO INTRACELLULAR AND SECRETED PROTEIN PRODUCTS EXPRESSED BY RECOMBINANT BACULOVIRUSES

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Key Words: Insect cells, Baculovirus, Unfolded protein response, Secretion, Expression analysis.

The baculovirus insect cell expression system has become a firmly established production platform in biotechnology. Various complex proteins, multi-subunit particles including veterinary and human vaccines are manufactured with this system on a commercial scale. Apart from baculovirus infected *Spodoptera frugiperda* (Sf9) cells, the *Trichoplusia ni* (HighFive) cell line is alternatively used as host organism. In this study, we explored the protein production capabilities of Tnms42 insect cells, a new derivative of HighFive, which is free of latent nodavirus infection. As a model system, a cytosolic (mCherry) and a secreted (hemagglutinin) protein were overexpressed in Tnms42 cells. The response of the host cells was followed in a time course experiment over the infection cycle by comparative transcriptome analysis (RNA-seq). As expected, the baculovirus infection per se had a massive impact on the host cell transcriptome, which was observed by the huge total number of differentially expressed transcripts (>14,000). Despite this severe overall cellular reaction, a specific response could be clearly attributed to the overexpression of secreted hemagglutinin, revealing limits in the secretory capacity of the host cell. About 400 significantly regulated transcripts were identified and assigned to biochemical pathways and gene ontology (GO) categories, all related to protein processing, folding and response to unfolded protein. The identification of relevant target genes will serve to design specific virus engineering concepts for improving the yield of proteins that are dependent on the secretory pathway.

GENERATION AND EFFICACY ASSESSMENT OF A CHIMERIC ANTIGEN E2-CD154 AS A MARKER CLASSICAL SWINE FEVER VIRUS SUBUNIT VACCINE PRODUCED IN HEK 293 AND CHO K1 MAMMALIAN CELLS

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The E2 glycoprotein is the major antigen that induces neutralizing and protective antibodies in CSFV infected pigs, thus a marker vaccine based on this antigen appears to be the most promising alternative to induce a protective immune response against CSFV. However, the structural characteristics of this protein state the necessity to produce glycoprotein E2 in more complex expression systems such as mammalian cells. In this study, we use a lentivirus-based gene delivery system to establish a stable recombinant HEK 293 and CHO K1 cell line for the expression of E2 fused to porcine CD154 as immunostimulatory molecule. In a first experiment, E2his and E2-CD154 were compared in an immunization trial. The average antibody titers in E2his immunized pigs was in the range of 30-40% of blocking and the average antibody titers for E2-CD154 are above 40% at day 14, meaning that the chimeric antigen is able to raise antibodies at positive levels in a shorter time. Additionally, the blocking rate of E2his vaccinated group in ELISA ranged between 66-88% and in the E2-CD154- vaccinated groups ranged between 86-92%, one week after booster immunization. The NPLA antibody titers also increased greatly. Later on, the protective capacity of purified E2-CD154 glycoprotein was demonstrated in a challenge experiment in pigs using a biphasic immunization schedule with 25 and 50 µg. The immunized animals developed neutralizing antibodies that were protective when the animals were faced to a challenge with 10^5 LD50 of ‘‘Margarita’’ CSFV highly pathogenic strain. No clinical signs of the disease were detected in the vaccinated pigs. Unvaccinated pigs in the control group exhibited symptoms of CSF at 3–4 days after challenge and were euthanized from 7–9 days when the pigs became moribund. These results indicate that E2-CD154 produced in recombinant HEK 293 and CHOK1cell line is a high quality candidate for the development of a safe and effective CSFV subunit vaccine. In the next steps, pilot and production scale, E2-CD154 expression levels should be increased in 10 to 50 fold, arriving to a very attractive productive platform for an implementation of a commercial subunit vaccine against CSF.

PRODUCTION AND CHARACTERIZATION OF HER2-DISPLAYING BUDDED VIRUS-LIKE PARTICLES AND THEIR POTENTIAL AS CANCER VACCINES

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Key Words: Human epidermal growth factor receptor 2 (HER2), baculovirus-insect cell expression vector system (BEVS), virus-like particles (VLPs), cancer vaccine.

Cancer biomarkers are often glycosylated membrane receptor proteins present on the cellular surface. In order to utilize such receptor proteins in designing specific and sensitive diagnostic tools or as immunogens for vaccination based treatments, they need to be expressed in their native conformation. However, membrane receptor proteins are notoriously difficult to produce due to their hydrophobic nature and complex structure. The human epidermal growth factor receptor 2 (HER2) is known to be up regulated in a number of cancers including breast cancer, lung cancer, gastric cancer and glioblastoma multiform and was therefore chosen as tumor antigen in our studies. Here we used the baculovirus-insect cell expression vector system (BEVS) to produce budded virus-like particles (VLPs) serving as a display platform for the antigen. VLPs displaying HER2 were produced in *Spodoptera frugiperda* (*Sf*9*)* insect cells and were purified by sucrose gradient ultracentrifugation. The number of secreted particles was quantified by nanoparticle tracking analysis. To confirm the presence and functionality of displayed HER2, VLPs were labeled with gold-conjugated antibodies, were analyzed by transmission electron microscopy and the ability to present native epitopes was tested through enzyme-linked immunosorbent assay (ELISA). Trastuzumab, an anti-HER2 antibody, showed significant binding to antigen displaying VLPs, which demonstrates the potential of this platform to display cell surface biomarkers in their authentic conformation. In the second part of the study, the efficacy of the aforementioned characterized VLPs as a cancer vaccine was investigated. BALB/c mice were injected intramuscularly with control VLPS and HER2-displaying VLPs in combination with two different adjuvants in a prime-boost regimen. As verified by ELISA, HER2-displaying VLP vaccines induced strong antibody responses when tested against recombinant HER2, with variability observed amongst the different adjuvant groups. For further characterization the antibody-dependent cell-mediated cytotoxicity (ADCC) potential of the induced antibodies will be investigated and vaccinated mice will be challenged with HER2 expressing tumors to test the potential of antigen-displaying VLPs as a cancer vaccine. Overall, using our strategy, many other membrane proteins including tumor antigens, immune cell markers and immune receptors could be expressed. These tools could further be instrumental in cancer vaccine design and diagnostics, as well as antibody selection and engineering.

RAPID FERMENTATION OPTIZATION FOR VACCINE DEVELOPMENT

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Key Words: Pichia pastoris, fermentation optimization, vaccine development.

Pichia pastoris is widely used to produce heterologous proteins including vaccines such as hepatitis B [1]. P. pastoris is capable of achieve high cell density during fermentation and has the ability to secrete recombinant proteins. Fermentation optimization can be a time consuming and laborious process that can cause delays in early vaccine development. To overcome this, we have made use of parallel automated scalable fermentation technology. Four single-use parallel small-scale fermenters (ambr250 modular) were used to rapidly screen and optimize fermentation parameters. A non-replicative rotavirus (NRRV, PATH) vaccine was expressed in P. pastoris. The methylotrophic yeast was grown in fed-bath mode using chemically defined media with glycerol as carbon source and induced with methanol. Intact mass spectrometry was used to determine the distribution of full length and truncated species at different induction times during fermentation. Zymogram analysis was used to detect potential proteases present in the fermentation supernatant.

The bioreactor operating parameters (pH, temperature, induction time and media composition) were optimized to improve cell growth and product yield. Resulting in product yields of ~ 1 g/L and purity levels of ~ 80% in the fermentation supernatant. Conditions were selected to reduce levels of truncation and increase production of full-length product. It was observed that temperature had little or no effect on mitigating product truncation; while fermentation pH and induction time had a greater effect, significantly reducing product truncation. In addition, zymogram analysis showed that levels of contaminating proteases in the supernatant were also affected by fermentation parameters and induction time. Cation exchange chromatography (CIEX) was used to purify the product directly from the fermentation supernatant showing that it is possible to integrate the up-stream process of fermentation with down-stream purification into a single procedure.

Single-use small-scale fermenters are useful for a rapid screening and optimization of fermentation parameters for vaccine development. These results show that the bioreactor operating parameters have a great effect on both product yield and quality and fermentation parameters can be optimized to reduce degradation of secreted products.

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CRISPR-dCAS9 FOR CONTROLLING BACULOVIRUS REPLICATION AND INCREASING PRODUCTION OF VIRUS-LIKE PARTICLES

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Key Words: IC-BEVS, CRISPR-dCas9, Virus-like particle, insect cell, baculovirus.

The Insect Cell – Baculovirus Expression Vector System (IC-BEVS) has proven to be a robust and efficient platform for the manufacture of recombinant proteins for virus-like particles (VLPs). However, IC-BEVS suffers from a general lack of developed genetic tools to permit rational genetic engineering to further improve efficiency and yields. Replication of the baculovirus is an inherent process of the infection cycle and may represent a significant diversion of cellular resources away from recombinant protein production. Although previous studies have reduced or eliminated the production of baculovirus by deriving various knockout mutants and complementing cell lines, production of recombinant proteins is typically equal to or lower than that of a replication-competent BEV. We believe that a reduction in endogenous BEV proteins should allow for increased expression of desired recombinant proteins.

To address baculovirus replication, we have developed and applied the CRISPR-Cas9 system for transcriptional repression. We have developed an Sf9 cell line constitutively expressing the *dcas9* gene and have successfully down-regulated the expression of a fluorescent protein (FP) reporter using transfection-based assays in this cell line. We have confirmed repression of the same FP reporter produced using immediate early, late, and very late promoters in IC-BEVS, and are currently scrutinizing targets to achieve transcriptional repression of endogenous genes encoded by *Ac*MNPV. Our aim is to target BEV genes in order to prolong the infection cycle and/or reduce baculovirus replication in order to further improve production of VLPs. We will then assess our system for improved production of model intracellular and secreted proteins, and finally model enveloped and non-enveloped VLPs.

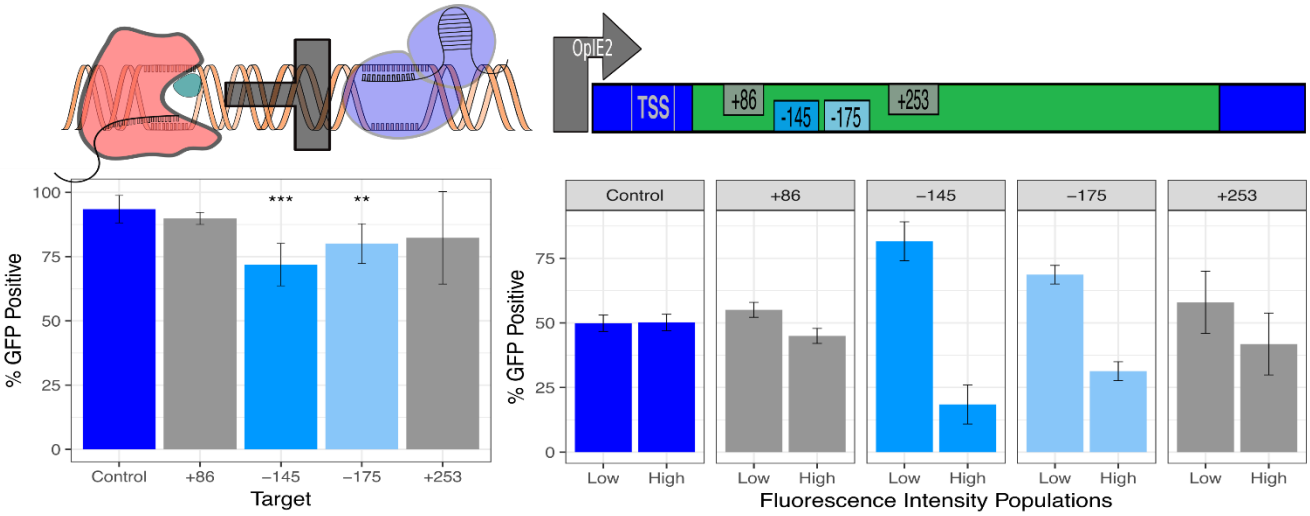

Figure 4 – Transcriptional repression of a fluorescent reporter gene in Sf9 cells using CRISPR-dCas9. Targets refer to the position of the protospacer in relation to the start codon, and strand (+ for coding strand, - for template strand). Low and high bins refer to the number of events below (low) or above (high) the median fluorescent intensity of the control.

PURIFICATION OF FLAVIVIRUS VLPs BY A TWO-STEP CHROMATOGRAPHIC PROCESS

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Key Words: virus-like particles (VLPs), yellow fever virus, zika virus, downstream processing, chromatographic purification

Flaviviruses are enveloped viruses with positive-sense, single-stranded RNA, which are most commonly transmitted by infected mosquitoes. Besides for example dengue viruses (DENV), which have been already for decades posing challenges to public health worldwide, zika virus (ZIKV) and yellow fever virus (YFV) are flaviviruses that have caused significant outbreaks in the last few years. Thus, based on our experience of expressing virus-like particles (VLPs) of several different flaviviruses in recombinant mammalian cells, this work focuses on the development of efficient chromatographic purification processes for zika and yellow-fever VLPs. ZIKV has been discovered in 1947, and since 2007 it has caused isolated outbreaks in Pacific Islands. However, in 2015 it was identified for the first time in Brazil and then quickly spread to over 60 countries between 2015 and 2016. Although most zika patients are asymptomatic, in a small proportion of adults ZIKV infection can cause Guillain-Barré syndrome, and in fetuses of infected women it frequently causes serious congenital malformations, especially in the central nervous system. Since it can be transmitted also by the sexual route and can persist for very long periods in body fluids (including sperm), the development of a vaccine is needed to prevent the spread of the virus to non-endemic countries and to prevent outbreaks to periodically occur in regions where the virus is already circulating.

Yellow fever virus is a highly lethal virus, which causes death in about 6-10% of non-vaccinated individuals. In past centuries, before the introduction of the current live-attenuated vaccine, 10% of the population of cities like Philadelphia (USA) and Barcelona (Spain) died in YF outbreaks. The current vaccine is very safe and provides life-long protection from a single dose. However, it can also cause fatal adverse effects in a small proportion of vaccines, and the egg-based production is limited in capacity. This latter fact led to worldwide vaccine shortages during an outbreak in Africa in 2016 and in Brazil in 2017-2018. Although the WHO introduced during the African outbreak in 2016 the use of a fractional (1/5) dose as an emergency measure to control outbreaks, even if using fractional doses of the current vaccine, shortage would be an issue if YF outbreaks spread and especially if it gets to be locally transmitted in Asia, where the mosquito vector is widespread.

In this work, a two-step chromatographic process was developed for the purification of zika and yellow fever VLPs from CHO- and HEK293-derived cell culture supernatant, building on previous experience acquired on the purification of yellow fever whole virus from Vero cell culture (Pato et al., 2014, doi: 10.1016/j.vaccine.2014.02.036). The initial clarification of the cell culture suspension was performed by centrifugation and/or filtration, followed by anion exchange chromatography and then a multimodal chromatographic step. The anion exchanger used was a Q membrane adsorber, due to its easy scalability, simplicity to handle, absence of diffusional limitations, and good performance at high flow rates for the capture of large molecules such as VLPs. This capture step allowed a high degree of concentration and an efficient DNA removal. In order to enhance HCP removal, a CaptoCore 700 multimodal column was used in a flow-through mode, allowing contaminants to be adsorbed while VLPs were excluded by size. Samples from all steps of the process were characterized by immunoassays, total protein determination, SDS-PAGE and Western blot.
The promising results obtained for zika and yellow fever VLPs indicate that this process could be potentially applied also to other flavivirus VLPs that we have been expressing in our lab, such as DENV1-4, SLEV, CPCV and ILHV. Overall, the presented downstream process could potentially represent a simple, robust and economic platform technology for the production of cell culture-derived recombinant flavivirus vaccines.

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EXPISF™: A CHEMICALLY-DEFINED BACULOVIRUS-BASED EXPRESSION SYSTEM FOR ENHANCED PROTEIN PRODUCTION IN SF9 CELLS

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Key Words: chemically defined, consistency, protein expression, baculovirus

The Baculovirus Expression Vector System (BEVS) is one of the major platforms for recombinant protein production and the last decade has become a preferred platform for vaccine development. Unlike mammalian expression systems that have long since transitioned to serum-free, chemically-defined culture media, relatively little innovation has taken place in insect expression systems, with insect cells continuing to rely on undefined, yeastolate-containing culture media that can exhibit significant lot-to-lot variability in terms of both cell growth and protein expression. Here, we present the development of a novel Sf9-based Baculovirus expression system based on a high-density, chemically-defined culture medium, a high-expressing Sf9 cell line, improved transfection reagent to faster generation of baculoviruses and expression enhancer that allow for consistent production of recombinant proteins with two-fold or greater improvements in protein titers compared to traditional BEVS workflows.

MEDIA FORMULATION TO SUPPORT THE GROWTH OF VERO CELLS IN SUSPENSION

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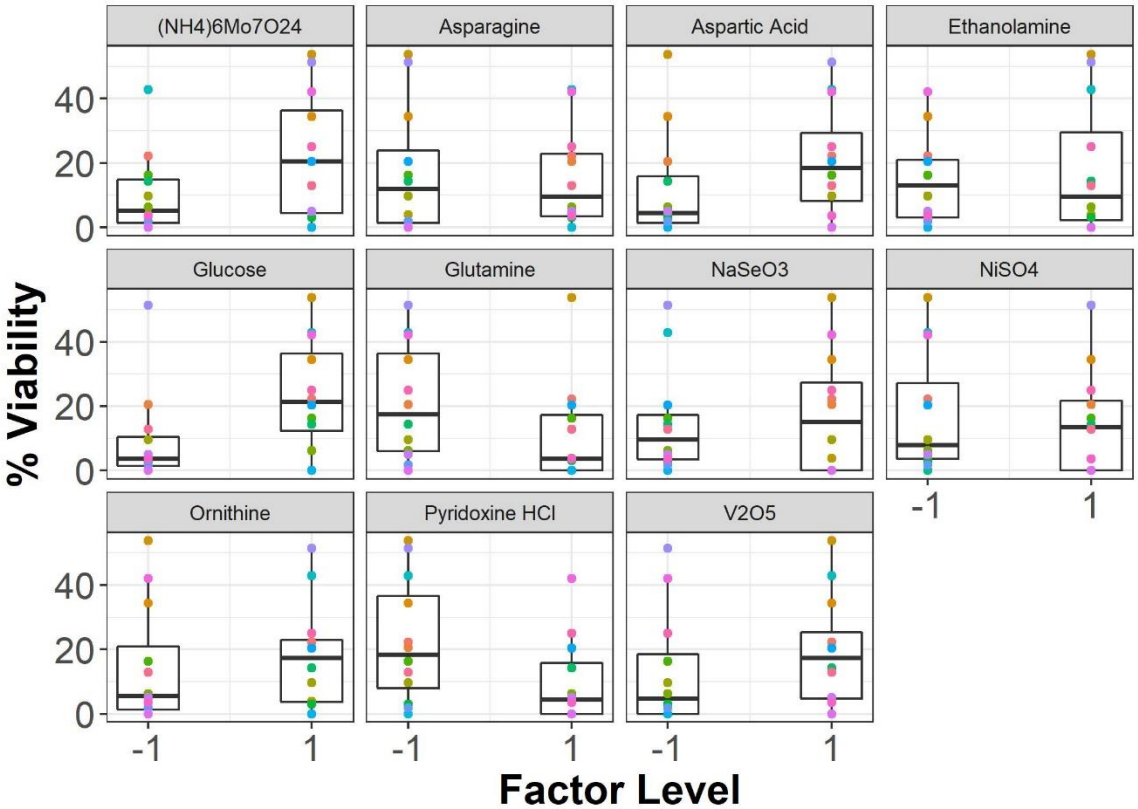
Key Words: Vero, Suspension, Serum-free, Media, Plackett-Burman, NMR

Vero cells are used in the production of many commercial vaccines but is limited due to their anchorage dependence. In order to scale up production, manufacturers have to use microcarriers for production using bioreactors or large rooms of roller bottles or cell stacks. These support matrices are essential for Vero cell growth and proliferation, but add substantial cost for vaccine production and limit the scalability. Using a design of experiments approach, media was that support the growth of Vero CCL-81 cells in suspension. First, a Plackett-Burman experiment was set up with 24 runs testing 23 factors using low calcium and magnesium DMEM/F12 as the base media. The factors were chosen to support suspension growth in serum-free media and could be split into four categories; amino acids, antioxidants, trace metals and vitamins, and growth factors. Cells were first adapted to serum free conditions using OptiPro SFM media in and then were gradually adjusted to the new various media formulations. L-glutamate and L-glutamine had a negative effect on cell viability when GlutaMax was already present in the media, while L-asparagine and L-aspartic acid were beneficial for suspension and suspension cell growth. Cells growing in suspension also benefited from increased levels of L-leucine, L-lysine, and L-methionine. The addition of rEGF and trace metals were essential for cell growth and high levels of selenium and ethanolamine ($3x10^{-4}$ mM and 0.041 mM, respectively) supported serum free growth. Further characterization of nutrient consumption patterns using 1D-1H NMR coupled with targeted profiling has aided in the refinement of media for enhancing the suspension phenotype.



STABILIZATION OF FREEZE SENSITIVE ADJUVANTED VACCINE FORMULATIONS TRHOUGH SPRAY DRYING

Figure 5 – Viability of suspension cells was after 4 days of culture was tracked. The percent viability is plotted against the factor levels of 11 compounds that had large effects on cell health.

Poster Number 55

WITHDRAWN

ACCELERATING THE MANUFACTURE OF GLYCOCONJUGATE VACCINES FOR PNEUMOCOCCAL DISEASE

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Key Words: *Streptococcus pneumoniae*, Glycoconjugate Vaccines, Protein Glycan Coupling Technology (PGCT), *Escherichia coli*

Streptococcus pneumoniae (S. pneumoniae) is one of the leading causes of invasive bacterial disease in children. In 2000 it contributed to 11% of deaths in children aged 1-59 months (1). Invasive pneumococcal disease can result in septicemia, meningitis and pneumonia. Two pneumococcal glycoconjugate vaccines were introduced in 2000 and 2010 in the USA. Deaths due to the serotypes included in these vaccines in children under 5 have fallen from 183 cases per 100,000 in 1998, to 11 per 100,000 in 2015.

The current gold standard glycoconjugate vaccine for immunization is Prevenar 13. It is the leading global vaccine product generating over $6 billion dollars in revenue in 2016. The cost per dose in the USA is over $100 with a total of four doses required in young children. Due to the complex manufacturing process for the vaccine the expense of the dose is driven up. The process involves separate fermentations for the thirteen serotypes and for the carrier protein component. Subsequent to this are stripping, purification, activation and chemical conjugation steps to make the vaccine and another series of purifications to make the final formulation. The chemical conjugation step requires personnel with a high level of experience and intricate knowledge of the reaction and is a limiting factor for new low income country manufacturers in entering this market.

The advent of Protein Glycan Coupling Technology (PGCT) has been an important development. This plasmid-based technology is able to produce glycoconjugate vaccines intracellularly in *E. coli* (2). As a result, there is no need for the chemical conjugation steps, meaning personnel do not need to have the niche skills currently required. Furthermore, the number of purification steps during the process are also reduced.

E. coli cells have been engineered with PGCT to produce a glycoconjugate vaccine of Serotype 4 of *S. pneumoniae*. Using this cell line, transition of *E. coli* growth from shake flasks into small scale bioreactors has been performed. Results show cell biomass is increased in bioreactors and volumetric productivity of cells is improved. It has also become apparent that any changes to the system need to be carefully considered. For example, comparison of two different proteins in this system has found one is more amenable to glycosylation indicating that choice of protein will have an effect on glycoconjugate production. Overall these experiments have demonstrated the scalability of PGCT and has laid the foundation for future optimization of the system.

The work presented here is using *S. pneumoniae* in a platform process for the production of pneumococcal conjugate vaccines. Here a new vaccine production technology is being optimized and scaled to increase product yields, and with a long term aim of reducing cost per dose of the vaccine.

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WITHDRAWN

DEVELOPMENT OF A VACCINE PRODUCTION PLATFORM FOR POULTRY DISEASES IN AFRICA: NEWCASTLE DISEASE VIRUS NON-REPLICATIVE ADENOVIRUS-VECTORED VACCINE

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Key Words: Vaccine production platform, Newcastle Disease Virus, bioprocess development, adenoviral vector, veterinary vaccine.

Poultry are a vital village livestock playing an important economic and nutritional role in the livelihoods of poor rural households in developing countries, including the Sub-Saharan Africa. Poultry production in Africa is threatened by infectious diseases such as Newcastle Disease (ND), which is highly contagious and endemic, with recurrent outbreaks that provoke heavy losses every year. ND is caused by the Newcastle disease virus (NDV), a negative-sense single-stranded RNA virus from the genus Avulavirus, family *Paramyxoviridae*. In particular, ND is one of the major problems in village chickens in most parts of Ethiopia where commercial poultry is routinely vaccinated with inactivated or live vaccines. Available ND vaccines are produced in specific pathogen free chicken embryonated eggs, whose supply is expensive and imported from Europe.

The development and execution of the present project, funded by the Canadian International Development Research Center and presently in its initial phase, aims towards the implementation at the National Veterinary Institute (NVI), Ethiopia, of a technological platform for the production of veterinary vaccines based on the development of recombinant non-replicating adenoviral vectors, using the human adenovirus serotype 5 (Ad5). The ND adenovirus vaccine proposed, expressing protective antigens from ND virus (NDV), will provide an efficient and cost-effective system to address the limitations associated with the current vaccines such as efficacy and virus shedding in flocks of vaccinated birds.

The key success factor of the project relies on the development of a robust and cost-effective production platform using serum-free suspension HEK293 adapted cells expressing maximized rAd5 product yields. This will be achieved by augmenting the production cell mass and the cellular productivity beyond cell densities of 6 million cells per mL. Critical parameters and operating conditions impacting the yield and quality of the Ad vaccine will be identified and elevated in a rational process operating strategy that will lead to high-cell density productive infection in bioreactors. Process development and scale-up will be followed by a downstream processing, evaluation of immunogenicity, formulation and stability assays, and protective capacity assessments after viral challenge in the target animals.

Recombinant adenoviruses have been generated carrying the NDV coding sequences for the fusion (F) or the hemagglutinin-neuraminidase (HN) proteins, and also for co-expression of both genes in a bicistronic construction. Phylogenetic analyses were primarily conducted to ensure a high degree of sequence identity of the genes cloned with the genoype of locally circulating strains. Recombinant protein expression was also designed and analyzed under different regulatory sequences aiming for selection of the most immunogenic variant. Following the initial phases of project execution, the subsequent steps will define the final parameters for high-cell density infection and rAd5 production for the animal studies.

Here we discuss in detail the completed and upcoming project steps as well as the different strategies implemented to achieve the set objectives supporting the main goal of sustainable technology transfer and capacity building of the NVI in Ethiopia.

FORMULATION AND STABILIZATION OF A RECOMBINANT HUMAN CYTOMEGALOVIRUS VECTOR FOR USE AS A CANDIDATE VACCINE FOR HIV-1

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Key Words: HIV vaccine, Cytomegalovirus, formulation, freeze-thaw, stability

Vaccination using Cytomegalovirus (CMV) vectors have recently shown promising results in conferring protection in non-human primates against SIV and Mycobacterium tuberculosis infection (1-3). Since CMV vectors can stimulate the production of high concentrations of systemic effector memory T-cells, CMV vectors (containing the appropriate insert) have the potential to clear SIV/HIV and Mycobacterium tuberculosis infection, provided administration occurs at the onset of infection (1, 3). Despite the promising animal data, CMV vectors are prone to potency loss (i.e., degradation) by freeze-thaw and storage at 2-8°C. In this study, we wished to develop formulations with increased freeze-thaw and liquid stability for a recombinant human CMV vector (rHCMV-1) for use in initial clinical trials including i) reduce vector potency loss to <0.5 log after 1 freeze-thaw cycle and ii) reduce vector potency loss to <0.5 log after 4 hours at 2-8°C storage. To achieve these goals, we screened a library of ~50 pharmaceutical excipients and evaluated their effect on vector potency after 3 freeze-thaw cycles or incubation at 4°C for several days. We found that certain additives completely protected rHCMV-1 against freeze-thaw mediated potency loss. With regards to liquid stability, we found certain additives slowed the rate of rHCMV-1 titer loss when stored at 4°C. After screening various excipient combinations, we evaluated three candidate formulations and benchmarked them against the bulk drug substance (BDS) formulation buffer and another published formulation (4). The candidate formulations were significantly more stable than the formulations used for benchmarking in terms reducing rHCMV-1 titer loss due to freeze-thaw and incubation at 4°C for up to 30 days. Despite providing greater stability than the current BDS formulation buffer, rHCMV-1 titer loss was still observed at 4°C as a function of incubation time, which suggests further stabilization (i.e., lyophilization) is likely necessary for longer term development. This study highlights the utility of empirical design of a liquid formulation of a live viral vector where freeze-thaw and short-term liquid storage are necessary.

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FORMULATION DEVELOPMENT OF A STABLE, ORALLY DELIVERED LIVE HUMAN NEONATAL ROTAVIRUS (RV3-BB) VACCINE CANDIDATE

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Key Words: Rotavirus, Gastroenteritis, Neonatal rotavirus vaccine, RV3-BB, intussusception

Rotavirus is the most common cause of gastroenteritis among children under 5 years of age leading to ~200,000 deaths in 2013.[1] Rotavirus-attributed mortality can be significantly reduced by promoting global implementation of rotavirus vaccination by vaccine dosage cost reduction and optimizing vaccine efficacy in low-resource countries. Furthermore, a rotavirus vaccine administered at birth could prevent neonatal mortality and reduce the risk of intussusception [2]. An oral human neonatal rotavirus vaccine candidate (RV3-BB) has been developed from the human neonatal rotavirus strain RV3 (G3P[6]) [2], and a recently published Phase IIb clinical trial showed RV3-BB was efficacious in preventing severe rotavirus gastroenteritis via a neonatal or infant schedule in Indonesia[2]. The overall goals of this project are to develop and implement commercially viable bulk and drug product manufacturing processes of a stable liquid formulation for oral delivery (without pre-neutralization) that is affordable in the developing world (Fig. 1). The consortium working on this program is sponsored by the Bill and Melinda Gates Foundation between Batavia Biosciences, Murdoch Children's Research Institute, BioFarma, and The University of Kansas.

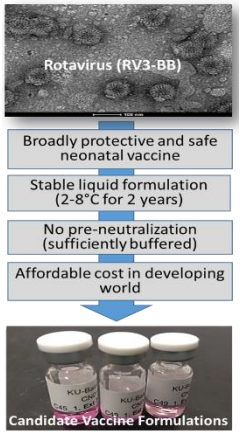

Fig. 1 – Considerations for the development of neonatal rotavirus vaccine (RV3-BB)

The formulation goals are to develop a refrigerator stable (i.e., 2 years, 2-8ºC) liquid formulation, based on the same RV3 strain, with no requirement to pre-neutralize gastric acid during administration. Analytical characterization of virus bulks and purified virus was performed in terms of potency, size, concentration, morphology, and physical stability. FFA and qPCR potency assays were developed and implemented for formulation development and stability evaluation. Optimization of various formulation parameters including pH and excipients to stabilize RV3 against various environmental stresses (thermal, agitation, and freeze-thaw) was performed. Excipient screening was first performed to evaluate the effect of individual excipients, followed by evaluation of various combinations. Rotavirus vaccines present a unique formulation challenge requiring careful balance of stabilizing the virus against low pH environments during oral delivery vs. long term storage stability. Accelerated and real-time stability studies with RV3 candidate formulations are in progress to elucidate the most stable formulation(s) that meet the aforementioned criteria (i.e., 2 year stability at 2-8ºC and withstand acid induced potency loss). Stability modelling studies based on extrapolation of accelerated and real-time stability data to predict long-term formulation stability are ongoing.

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CASE STUDY: SINGLE-USE PLATFORM FOR COMPLETE PROCESS DEVELOPMENT AND SCALE-UP OF AN ADENOVIRUS

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Virus production for vaccines and gene therapy relies heavily on adherent cell culture based processes – typically trays, roller bottles or microcarriers. The introduction of the iCELLis® fixed-bed bioreactor and single-use purification operational units provides a full solution for rapid process development at small-scale in the iCELLis Nano bioreactor with direct, predictable process transfer to the large-scale iCELLis 500 bioreactors. The iCELLis 500 bioreactor provides a fixed-bed with 66 - 500 m² of surface area for cell growth. A 500 m² fixed-bed is equivalent to 5,882 roller bottles of 850 cm² or 278 HYPERStack 36. Coupled with other single-use upstream and downstream technologies, the iCELLis bioreactor and purification membranes provide a complete single-use platform for virus production. We have performed the complete process development and scale up of an adenovirus (Ad5) process using HEK-293 cells. The reference process using 2D flatware was transferred, optimized and scaled-up into the iCELLis Nano bioreactor (surface areas of 0.53 - 4.0 m²). Based on results in the iCELLis Nano bioreactor and virus requirements, the 66 m² large-scale iCELLis 500 fixed-bed bioreactor was selected. In order to simplify the seed train, the fully-closed, single-use Xpansion® multiplate bioreactor was used. The upstream yield from the iCELLis 500 66 m^2 bioreactor was 1.04 x 10^{16} of Infectious Units (IFU). In this study we optimized and developed adenovirus purification manufacture processes using Mustang® Q with bind/elute strategy managed to reduce significantly the impurities such as HCP and HC-residual DNA and enriching full vs. empty capsid (>90%). The eluted Ad5 from Mustang Q membrane is immediately processed through the UFDF for further concentration and buffer exchange to final virus formulation buffer. Final purified product was then sterile filtered and vialed for potency studies. Downstream processing utilized single-use systems with 62% recovery for a final purified yield of 6.42 x 10^{15} IFU. Analytical characterization of the virus met specifications and *in vivo* GLP toxicology testing results were comparable to material produced using the reference process. Scalable upstream and downstream strategies for production and purification of virus based product as such described here offers a fast-to-market, more cost effective alternative to traditional processes. We will review the iCELLis bioreactor platform and downstream purification platform and present as a case study the process development and scale up of the complete adenovirus (Ad5) process.

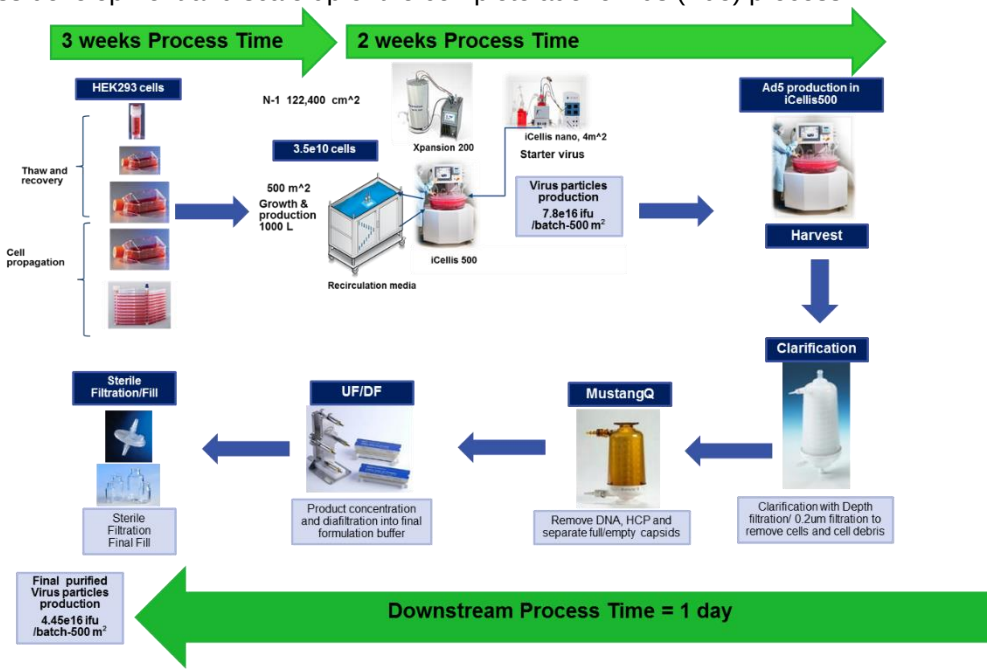

Figure 1: Virus-based product production and purification workflow process

DEVELOPMENT AND CHARACTERIZATION OF A MURINE HEPATOMA MODEL EXPRESSING HEPATITIS C VIRUS (HCV) NON-STRUCTURAL ANTIGENS FOR EVALUATING HCV VACCINES

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Key Words: Hepatitis C Virus, Vaccine, Non-Structural Antigens, Hepatoma Model

Hepatitis C (HCV) is a highly prevalent blood-borne virus with infection of 2-3% of world population and high rate of chronicity (>70%) leading to chronic hepatitis, which often progress to cirrhosis and hepatocellular carcinoma. HCV- specific immune responses consisting of CD4 and CD8 T cells and virus neutralizing antibodies have been shown to eliminate HCV infections in humans and chimpanzees. Therefore, vaccines that can induce potent and durable anti-HCV T and B cell responses may have the potential to clear chronic HCV infections. A number of HCV vaccines have been tested clinically with limited success. One of the major limitations in developing effective HCV therapies is the lack of effective and reliable animal models due to the narrow host range of the HCV virus. The study described herein reports the generation of a murine hepatoma cell line expressing HCV non-structural proteins and its use in a metastatic tumor setting to test anti-tumor efficacy of bacterial and viral vector vaccines expressing the HCV non-structural genes. HCV-recombinant hepatoma cells formed large solid-mass tumors when implanted into syngeneic mice, allowing the testing of HCV vaccines for immunogenicity and anti-tumor efficacy. Using this model, we tested the therapeutic potential of recombinant anti-HCV-specific vaccines based on two fundamentally different attenuated pathogen vaccine systems - attenuated Salmonella and recombinant adenoviral vector based vaccine. Attenuated Salmonella secreting HCV antigens limited growth of the HCV-recombinant tumors when used in a therapeutic vaccination setting. The inhibition of tumor growth by Salmonella vector-based vaccines was significantly reduced in mice co-injected with an anti-CD8 antibody, suggesting a role by CD8+ cells in the vaccine efficacy. The model was also used to compare replication deficient and replication-competent but non-infectious adenoviral vectors expressing non-structural HCV antigens. Results showed overall greater survival and reduced weight loss with the replication-competent vector compared to the non-replicating vector. Our results demonstrate the novel recombinant murine hepatoma model expressing HCV non-structural antigens as a useful model for evaluating therapeutic vaccines against HCV. Vaccines that are capable of inducing potent anti-HCV immune responses that are capable of controlling aggressive and metastatic tumor growth in this model would likely have the potential to control chronic viral infections such as HCV. This novel approach is particularly interesting for the development of therapeutic vaccines.

CASE STUDY: RAMAN IMPLEMENTATION FOR PROCESS LIFECYCLE MANAGEMENT IN FERMENTATION BASED PROCESSES

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Key Words: PAT, RAMAN, spectroscopy.

Existing metabolite analyzers for several inline fermentation based vaccine processes are becoming obsolete in upcoming years. Analyzers are used to support classified parameters and attributes as well as characterization of the fermentation processes. A PAT solution is preferred as a replacement as it would allow for enhanced process understanding and control. Raman spectroscopy has been aligned as a core technology for implementation with varying challenges based on media, organism, processing parameters and attributes being measured. Implementation requires a highly collaborative approach across functions and sites to ensure effective implementation with no interruption to supply. Using a standardized approach to Raman model development and validation, robust models have been developed for 2 product lines with implementation scheduled over the next three years.

ADAPTATION OF VERO CELLS TO SUSPENSION CULTURE AND RABIES VIRUS PRODUCTION ON DIFFERENT SERUM FREE MEDIA

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Key Words: Vero cells, suspension adaptation, rabies virus

Vero cells are nowadays widely used in the production of high quality vaccines for humans and animals. They are considered as one of the most productive and flexible continuous cell lines available for vaccine manufacturing. However, these cells are anchorage dependent, which greatly complicates upstream processing and process scale-up. Moreover, there is a recognized need to reduce costs of vaccine manufacturing in order to develop vaccines that are affordable worldwide. The use of cell lines adapted to suspension growth contributes to reach this objective.

The current work describes the adaptation of Vero cells to suspension culture in different serum free media according to multiple protocols. Then, the suspension adapted Vero cells (VeroS) were infected with rabies virus (strain LP-2061).

In addition to IPT-AFM (an in-house animal component free medium described in Rourou et al. 2009a; 2009b and 2014), five commercial formulations were analyzed for the establishment of serum free culture of VeroS. The cultures were performed in erlenmeyer flasks at 37ºC, 5% CO2.

The Cell doubling (CD) for VeroS adapted to IPT-AFM was 2,1$\pm$0.7; the average specific growth rate (μ) reached 0,016$\pm$ 0,003 h^{-1} and the maximum cell density (Xmax) amounted to 2,16$\pm$0,9x10^6 cells/mL. Through adaptation, the cells behave differently in the different media. A comparative study was performed and IPT-AFM showed promising results.

Kinetics of rabies virus replication in VeroS cells grown in IPT_AFM showed that rabies virus was able to replicate in VeroS cells and to achieve a virus titer of 6x10^7 FFU/mL at day 3 post infection.
These data show that the VeroS cell line can be considered as a suitable cell line for large scale rabies virus production.

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VIRUS-LIKE PARTICLES: A FLEXIBLE PLATFORM FOR UNIVERSAL INFLUENZA VACCINE DEVELOPMENT

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Key Words: Virus-like particles, *Pichia pastoris*, Tandem core, Hepatitis B core protein (HBcAg)

Human influenza remains a global public health threat, namely due to its evolutionary adaptability, which hinders effective prevention. Vaccination is currently the predominant tool in the prevention of infectious disease. However, current production methods for influenza vaccines are not only logistically inadequate in the face of a pandemic, but also rely on targeting two surface proteins on the influenza virus, which are prone to antigenic drift. As a consequence, a new vaccine needs to be developed for each new seasonal epidemic. Additionally, the vaccine strain needs to be selected around eight months prior to administration and can often be mismatched leaving the population unprotected. A 'universal' vaccine, effective irrespective of the surface proteins, would be desirable to offer cross-protectivity across strains.

Tandem core virus-like particles (VLPs), expressed in methylotrophic yeast *Pichia pastoris,* are an exciting alternative to current manufacturing methods. VLPs, due to their inherent safety profile and advances in genetic engineering, have excellent potential both as standalone vaccines for the virus from which they are derived, or as platforms for the display of foreign antigens. The hepatitis B core antigen (HBcAg) is able to spontaneously self-assemble, forming icosahedral particles that are inherently immunogenic. Moreover, the HBcAg is capable of carrying antigen inserts in the major insertion region (MIR) which are displayed on the particle surface.
In order for VLPs to be considered a viable alternative, their bioprocessing must be optimized. Currently, various issues are at play including problems with formation, solubility and immunogenicity, often clone dependent. In this work, two genetically linked HBcAg monomers, carrying different inserts in the MIR, were used to study the effects on fermentation efficiencies using two different induction strategies. Rationalizing an induction strategy would enable the development of an efficient process to produce and purify VLPs. Results indicate that increased biomass is not always synonymous with increased protein expression. Moreover, protein expression and solubility appear to be linked with the complexity of the inserts displayed on the VLP surface.

The aim of this work is to improve the bioprocessing of VLPs in a microbial expression system, using tandem core technology. This proposed method is cheap and rapidly scalable, reduces the cost per dose and eliminates the long production timelines associated with current manufacturing. The very nature of VLPs and the comparable ease of production would enable this to be promoted as a platform process, for a myriad of disease targets.

CHARACTERISTICS OF rVSV-ZEBOV PRODUCTION KINETICS IN HEK293 AND VERO CELLS

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Key Words: rVSV-ZEBOV, vaccine, cell culture, virus, bioprocess

The vesicular stomatitis virus (VSV) can be used as an effective vaccine platform, inducing both cellular and humoral immunity. Because VSV infections of humans are mostly asymptomatic, recombinant VSV (rVSV) can be used as a platform to safely deliver and express foreign antigens. This research study focusses on cell culture production of an rVSV expressing the Ebola virus glycoprotein on its surface (rVSV-ZEBOV). This virus has been demonstrated to be safe to administer to humans. In addition, recent results of a human phase III clinical trial showed that this vaccine can efficiently protect against Ebola virus infection. However, limited data is available in the literature about the growth characteristics of this virus during the production process. In our study, we investigated the influence of multiplicity of infection (MOI), time of infection (TOI), time of harvest (TOH), media components and temperature on the viral titer (TCID50/mL, ddPCR) of rVSV-ZEBOV produced from cell culture. Results are compared between the standard production in the Vero cell line and in a suspension-adapted HEK293-based cell line without serum.

LASER FORCE CYTOLOGY FOR RAPID QUANTIFICATION OF VIRAL INFECTIVITY

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Key Words: Viral infectivity, process analytical technology, bioreactor

The quantification of viral infectivity is an integral step at multiple stages in the process of virally producing recombinant protein, studying the mechanism of viral infection, and developing vaccines. Accurate measurements of infectivity allow for consistent infection and expansion, maximum yield, and assurance that time or environmental conditions have not degraded product quality. Traditional methods to assess infectivity, including the end-point dilution assay (TCID50) and viral plaque assay, are slow, labor intensive, and can vary depending upon the skill and experience of the user. Application of Laser Force Cytology (LFC) for the rapid detection and quantification of viral infection will be presented and discussed for several viral systems in the context of improving the development and production of vaccines. LumaCyte's Radiance™ instrument is an automated cell analyzer and sorter that measures the optical force, size, shape, and deformability and captures images of single cells. By measuring the intrinsic properties of single cells, cellular changes due to viral infection can be rapidly and objectively quantitated. LFC is very sensitive to agents that perturb cellular structures or change biochemical composition. High quality viral infectivity measurements can be made in a fraction of the time, labor, and cost of traditional assays such as plaque or endpoint dilution. For in-process automated bioreactor monitoring, infectivity can be measured by Radiance in near real-time throughout the process, allowing critical feedback control and optimization. The measurement speed and data quality of LFC / Radiance serve to enhance vaccine development, process optimization/scale-up, and manufacturing to ultimately improve the delivery of vaccines to patients.

FULLY AUTOMATED HIGH-THROUGHPUT PROCESS DEVELOPMENT FOR THE NOVEL PURIFICATION OF ROTAVIRUS VACCINES

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Key Words: Mixed-mode chromatography, Tecan automation, High-throughput process development, P2-VP8 rotavirus vaccine

Downstream processing of biopharmaceuticals, such as immunoglobulins, recombinant proteins and protein-based vaccines, traditionally requires multiple purification steps that can introduce cost and time-related limitations. To avoid these, alternative purification strategies are sought involving novel and efficient processes. Mixed-mode chromatography based unit operations can achieve these by making use of their multimodal functionality. This adheres to the goal of the Ultra-low cost Transferable Automated Platform for Vaccine Manufacture (ULTRA) project and its ultimate aim to deliver vaccines with a cost of goods less than 15 cents per dose.

Herein, we show the application of mixed mode chromatography for the purification of a P2-VP8 (PATH) rotavirus antigen vaccine expressed in *Pichia pastoris* (MIT, MA, USA; J. Christopher Love). A high-throughput workflow was adopted for the development of the purification step which employed microscale chromatography with miniature columns. This was implemented on an automated liquid handling station (Tecan Evo 200). The performed scouting studies focused on combinations of different binding and elution conditions along with multiple resins and selectively accessing the ion exchange and hydrophobic integration mechanisms of the resins. Therefore, a wide range of conditions were assessed with walk-away automation. Conditions leading to high yields and purities were then scaled-up for verification purposes. This provided further evidence for the good scalability properties of the miniature chromatography column technique.

The results from this study demonstrate that mixed mode chromatography can potentially lead to the establishment of a highly desirable, one-step purification of rotavirus vaccine upon further optimisation. Hence, downstream processing in rotavirus vaccine production can be de-bottlenecked with systematic process development activities leading to significantly improved whole process efficiencies. The utility of this method is that it has generated reproducible and scalable data with reduced sample requirements to just a few millilitres very early on in the development process. More broadly, this high-throughput methodology will enable the early purification screening of multiple vaccine candidates and enable their selection for further development based on ease of processing.

AFFINITY RESIN SCREENING FOR OPTIMAL DSP – APPLICATION TO ROTAVIRUS VACCINE PRODUCTION

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Key Words: Down-stream processing, affinity chromatography, resin screening, process optimization, high throughput.

A crucial step in the down-stream processing (DSP) of recombinant vaccine production is the correct choice of affinity chromatography resins for product purification. All too often vaccine DSP protocols are based on methodologies that worked to allow development of candidate products to proof of concept and clinical trial rather than being optimized for efficiency and cost. This “what worked” approach often leads to increased production costs. Our aim here, as part of a Bill and Melinda Gates funded ULTRA project, is a more systematic approach to resin choice and DSP development that will enable low cost of goods of 15cents/dose or less. Ideally, this approach should be incorporated into the early stages of vaccine development.

To achieve this we have implemented a resin screening protocol based on the use of 96 well filter plates where each well contains chromatography resins. This approach allows for the rapid screening of large numbers of potential affinity resins and bind/elute conditions. It has the advantage that it can be operated either fully automated (using TECAN robotic platforms) or manually using relatively simple laboratory equipment. It is therefore quite feasible to incorporate such an approach into DSP development irrespective of whether development is being carried out in a well-funded or resource-limited environment. This approach allows for a saving of many months in process development time along with significant savings in resources compared to a conventional resin and bind/elute condition screening using individual columns.

We have applied this approach to the screening of affinity chromatography resins for DSP of two non-replicative rotavirus (NRRV) vaccines. Bind/elute characteristics are determined by monitoring OD_{280nm} for total protein levels, OD_{260nm} for nucleic acid levels and specific product levels by antibody detection and finally SDS-PAGE. We have screened 19 potential resins for use in hydrophobic interaction (HIC), cation exchange (CIEX) and multimodal (MM) chromatography. Initial rounds of screening allowed exclusion of four HIC resins as completely unsuitable. Bind/elute conditions were further investigated for the remaining resins leading to the choice of optimal resin-bind-elution combination for each affinity class. These were scaled up to for use in column chromatography.

Using the data from the 96 well plate studies it proved possible to predict DSP protocols that can be used in NRRV purification by HIC, CIEX or MM chromatograph. These results further demonstrate which of these can potentially be incorporated into an integrated production process.

RAPID VIRUS TITRATION USING FLOW CYTOMETRY

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Key Words: Flow cytometry, virus, titration, granularity, bioprocess development .

Rapid high throughput virus titration methods are essential for facilitating continuous process monitoring and rapid decision making in viral bioprocess development. In spite of repeated efforts to address this need, the industry continues to reply on well understood and trusted plaque assays and end point dilution assays, or variations thereof. Together with the University of Waterloo, we have developed a flow cytometry based assay that is able to give infectious virus titers in a fraction of the time as compared to conventional virus titration assays.

The developed method utilizes the phenomenon of increased granularity in cells after virus infection, with the increase in granularity proportional to the multiplicity of infection of the virus. The assay has been adapted to a 96 well plate format which, in combination with the use of a flow cytometer with an automated sampler, results in a high throughput assay with much reduced operator effort as compared to traditional assays.
Two different cell and virus systems have been examined using this assay. Assay variations in both systems were measured to be ~20%, and assay accuracy was highly comparable to traditional "gold standard" assays such as the plaque assay. Assay analysis was found to be simple and amenable to automation through the use of R scripts. Operator effort was reduced by approximately half per sample, and the assay time was reduced by 75%, when compared to traditional assays. In addition, the simplicity of the assay greatly reduces operator training time.

Studies by other groups provide confidence that the phenomenon of increased cell granularity with virus infection is present in several virus-cell systems. Therefore, the developed method has great potential to be used as a routine high throughput screening technique for a wide range of viruses.

HIGHLY EFFICIENT INFLUENZA VIRUS PRODUCTION: A MDCK-BASED HIGH-CELL-DENSITY PROCESS

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Key Words: vaccine manufacturing, influenza virus, high-cell-density, MDCK suspension, semi-perfusion

Seasonal vaccination campaigns for influenza in developed and developing countries create a massive demand for 500 million (2015) vaccine doses every year [1]. Besides egg-based vaccine manufacturing, production platforms based on animal cell culture increasingly contribute to this overall growing market. In order to intensify cell culture-based influenza virus production, high-cell-density (HCD) cultivation of suspension cells can be applied to improve virus titer, process productivity and production costs [2]. For process optimization and evaluation of HCD conditions, cells cultivated using semi-perfusion approaches in small shakers can be used as a scale-down model for bioreactors operating in full perfusion mode [3].

In this study, a previously developed MDCK suspension cell line [4] was adapted to a new serum free medium [5] to facilitate higher growth rate, cell density and virus titer both in batch and in HCD. Therefore, MDCK cells cultivated in Smif-8 medium were slowly adapted to a new cultivation medium (Xeno™) by stepwise increasing the Xeno content. Fully adapted cells were cultivated in shaker flasks to evaluate the performance of influenza A virus production in batch and HCD. Cell densities exceeding $2 \cdot 10^7$ cells/mL were achieved in shakers using semi-perfusion, where cell free medium was manually replaced with fresh medium. Volume and time interval of media replacement were chosen to achieve a constant cell-specific perfusion rate of 2.5 pL/(cell h). Cell cultures were infected with influenza virus (A/PR/8/34 H1N1 RKI) with trypsin addition. Cell count, viability, main metabolites and virus titer (HA-assay & $TCID_{50}$) were monitored pre and post infection.

Medium adaptation resulted in a MDCK suspension cell line with morphological, growth, and metabolic characteristics different from parental cells. Cells fully adapted to Xeno medium were growing to higher cell densities ($1.4 \cdot 10^7$ vs $6 \cdot 10^6$ cells/mL) with higher specific growth rate (μ_{max}: 0.036 vs 0.026 1/h), cells were bigger (15-16 vs 13-14 µm) and grew without aggregate formation. Due to higher cell densities at time of infection, virus titers up to 3.6 $\log_{10}$(HAU/100µL) were reached. In semi-perfusion, adapted MDCK cells were grown up to $6 \cdot 10^7$ cells/mL, however, maximum virus titer and productivity were observed with $4 \cdot 10^7$ cells/mL. In multiple harvests, very high virus titer exceeding 4 $\log_{10}$(HAU/100µL) and up to $9 \cdot 10^9$ virions/mL ($TCID_{50}$) were measured, which corresponded to an accumulated titer of 4.5 $\log_{10}$(HAU/100µL). Cell-specific virus titer was similar or higher compared to the reference batch infections, depending on perfusion and infection strategy.

Overall, results in this semi-perfusion scale-down model for influenza A virus production suggest a highly efficient and productive upstream process for influenza virus production, with an up to six-fold improved space time yield compared to batch mode.

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A CLINICALLY VALIDATED *DROSOPHILA* S2 BASED VACCINE PLATFORM FOR PRODUCTION OF MALARIA VACCINES

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Key Words: Malaria, pfs25, vaccine, clinical trial

Drosophila S2 insect cell expression is less known than the extensively used *Spodoptera* or *Trichoplusia ni* (Hi-5) insect cell based Baculovirus expression system (BEVS). Nevertheless it has been used in research for almost 40 years. The cell line was derived from late stage *Drosophila melanogaster* (Fruit fly) embryos by Schneider in the 1970s, who named the cell line *Drosophila* Schneider line 2 (synonyms: S2, SL2, D.mel. 2). The system has been widely applied to fundamental research, where the availability of the whole genome sequence of *Drosophila melanogaster* (1, 2) and the S2 cells' susceptibility to RNA interference methods (3, 4) have enabled genome wide RNAi screening and whole genome expression analysis techniques to be used to great effect. S2 cells have proved to be highly effective for the production of proteins from a great variety of protein classes (5), such as: viral proteins, toxins, membrane proteins, enzyme, etc. Recent publications have also shown the strength of the S2 system in expression of Virus Like Particles (VLPs) (6).
ExpreS^{2}ion has developed the ExpreS2, *Drosophila* S2 platform to achieve improved yields for difficult to express proteins. Furthermore, several technologies have been developed to improve the ease of use of the system, as well as enable fast and efficient screening of multiple constructs.
S2 based production processes for two malaria vaccine clinical trails with The Jenner Institute, Oxford University (Rh5 (7,8), blood-stage malaria) and Copenhagen University (VAR2CSA (9) pregnancy associated malaria) have been developed. The placental malaria vaccine is currently in a phase Ia trail in Germany, and a Phase Ib trial in Benin. The blood-stage malaria vaccine is currently in Phase IIa trial and is expecting results by the end of 2018.

Several transmission-blocking candidates have been identified over the years with some of the most prominent being pfs48/45, Pfs230C and Pfs25(10). Other vaccine targets focus on blood-stage malaria such as Rh5, PfRIPR and CyrPA. We will present data on the development of a high producing Pfs25 monoclonal cell line and the purification from said cell line,as well as expression data on a range of other malaria vaccine targets.
This present the clinically validated ExpreS2 platform as a complete system for a wide range of malaria targeting vaccines.

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TECHNOLOGY TRANSFER FOR LOCAL VACCINE PRODUCTION IN ARGENTINA

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Key Words: Technology transfer, recombinant antigens, fill-finish capacity, influenza vaccine.

Technology transfer and local production can be an effective and sustainable strategy to access newer vaccines in developing countries. The influenza pandemic in 2009 highlighted the importance of having a steady supply of vaccines to assure a controlled public-health strategy. To address these issues, Sinergium Biotech S.A. developed an innovative public-private partnership to transfer technology from multinational companies for local vaccine manufacturing. In doing so, it aimed to introduce long-lasting technology in Argentina, to support the steady supply of critical vaccines, and to generate regional exporting capacity. To date, the company has built a green field facility and has successfully completed three technology transfers from worldwide recognized technology providers, establishing state-of-the-art formulation and fill-finish capacity. Sinergium Biotech currently supplies Influenza, Pneumococcal and HPV vaccines (approximately 18 million doses per year) from its current facility in the Buenos Aires suburbs to the Argentinian Ministry of Health. In 2016, the company started a technology transfer to produce the FDA approved recombinant Influenza Vaccine Flublok® locally, including the production of the monovalent antigens. To that end, Sinergium recently started the construction of a new facility that will produce recombinant antigens using the baculovirus expression system (BEVS). The Sinergium Biotech development laboratory is currently working on a downscale process manufacturing model and analytical methods which are critical to support the future manufacturing activities.

REAL-TIME STABILITY OF A HEPATITIS E VACCINE (HECOLIN®) DEMONSTRATED WITH POTENCY ASSAYS AND MULTIFACETED PHYSICOCHEMICAL METHODS

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Key Words: Neutralizing epitope, Hepatitis E vaccine, *In vivo* mouse potency, *In vitro* relative potency (IVRP), Real-time stability

The first prophylactic vaccine against hepatitis E virus (HEV), Hecolin®, was licensed in China. Recombinant p239 virus-like particle (VLP) is its active component with dimeric protein as the basic building block harboring the immuno dominant and neutralizing epitopes. The real time and real condition stability of the prefilled syringes for the vaccine was demonstrated using both in vivo mouse potency and in vitro antigenicity assays. A total of 12 lots of Hecolin® were assessed with a set of assays after storage at 2-8 °C for 24 months. The particle characteristics of p239 VLP recovered from the aluminum-containing adjuvant was assessed with different methods including analytical ultracentrifugation, high performance size exclusion chromatography and transmission electron microscopy. The thermal and conformational stability of the adsorbed antigen was assessed using differential scanning calorimetry. The protein integrity of the recovered p239 antigen was demonstrated using SDS-PAGE with silvering staining, LC-MS and MALDI-TOF MS. Most importantly, the binding activity to the neutralizing antibody or vaccine antigenicity was measured using an epitope-specific and real-time SPR assay and a monoclonal antibody-based sandwich ELISA. Taken together, the overall good stability of the Hecolin® prefilled syringes was demonstrated with unaltered molecular and functional attributes after storage at 2-8 °C for 24 months.

EVALUATING THE EFFECT OF FORMULATION ON THE UPTAKE OF A ZIKA SUBUNIT VACCINE CANDIDATE BY ANTIGEN-PRESENTING CELLS.

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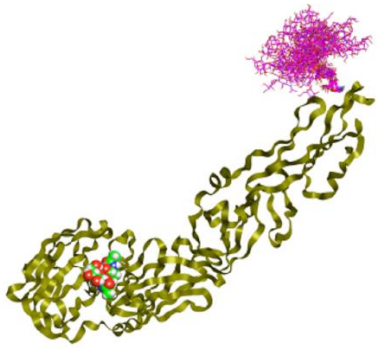
Key Words: Zika vaccine; Zika envelope protein; Alhydrogel; Liposomes; Antigen presenting cells

A major issue with vaccination for Zika, Dengue and other flaviviruses is the potential for antibody-dependent enhancement (ADE) of disease, caused by the generation or boosting of infection-enhancing antibodies. To address this concern, a subunit vaccine is being developed against the Zika virus using a modified version of the envelope protein as the antigen which has been modified with glycan residues to mask the fusion loop region of the protein (Figure 1), which is a cross-reactive and immunodominant site strongly implicated in the generation of antibodies capable of ADE. With this subunit vaccine approach, there is a need to formulate with an appropriate adjuvant to enhance the immunogenicity of the modified envelope protein. In this study we have evaluated a range of adjuvants using flow cytometry and fluorescence microscopy and have determined the relative uptake by human Antigen-presenting cells (APCs). Various combinations of clinically acceptable adjuvant materials: Alhydrogel®, 3D-(6-acyl) PHAD™ (a synthetic analogue of MPL) and QS21, were tested using liposomal formulations. In addition, the modified Zika envelope protein was compared to that of wild type Zika antigen, similarly formulated.

A further, more-general, issue in human vaccine development is the development of human-relevant test systems that might be used to better predict immunogenicity of vaccine antigens in combination with adjuvant formulations. To this end, dendritic cells were differentiated from human peripheral blood mononuclear cells (PBMCs) using a cytokine cocktail and matured with lipopolysaccharide (LPS). These differentiated dendritic cells were used as antigen presenting cells (APC) for antigen uptake performance evaluation. In brief, the cells were incubated with the vaccine formulations and antigen uptake was evaluated using flow cytometry and immunofluorescence. The antigen was detected using a fluorescently labelled anti-Zika envelope protein monoclonal antibody following cell permeabilization, and where applicable Alhydrogel uptake was evaluated using the aluminium specific fluorescent probe lumogallion. Uptake efficiency was evaluated in a time course fashion and at various amounts of antigen/adjuvant quantities. Fluorescent signal was correlated with the amount of antigen incorporated in the APCs. This approach allowed to compare the efficacy of multiple vaccine formulations in a human-relevant biological environment. The results demonstrated that all formulations were internalized by the APCs with equivalent uptake of the modified envelope protein and the wild type protein. Moreover, the liposomal formulations without Alhydrogel were internalized at a slower rate than those comprising liposomes alone.

Figure 1: Ribbon diagram showing the modified envelope protein with attached glycan.



IMPLICATIONS OF MDCK CELL HETEROGENEITY IN CELL-BASED INFLUENZA VACCINE PRODUCTION

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Key Words: MDCK cells, Cell heterogeneity, Clonal variability, Influenza vaccine, Transcriptome

Influenza is a global public health issue that causes serious illness with high mortality rate. Currently, Madin-Darby canine kidney (MDCK) cell culture-based influenza vaccine production moving up to the front as an inexorable trend for the substitution of egg-based vaccine production, owing to its high degree of flexibility and scalability. However, MDCK cells are a continuous cell line and comprise a heterogeneous pool of non-clonal cells that differ in morphological as well as functional features in influenza virus production. The impurity of cell population may lead to fugacious tendency in virus production, and long-term culture may bring potential risk of unstable viral production or vaccine quality as cells in MDCK subclonal population may encounter unexpected manifestation of chromosomal rearrangement, loss of the virus susceptibility, or reduction of the virus partials packaging capability during the culture. Although many details of the influenza virus life cycle have already been unraveled, little is known about the ability of subclones in virus infection, intracellular replication, and virus release during viral vaccine production process. With the widely utilizing of omics-based approaches and progressively accumulating of omics database, transcriptome profile analysis will be a powerful strategy to explore the mechanism of cell heterogeneity, providing great significance for the development of robust virus producing cell line and robust virus production process. This work aims to explore a deeper understanding on the MDCK cell heterogeneity used in influenza virus production. For this purpose, a MDCK cell line that has been extensively used in industrial production was subcloned and examined for the influenza virus productivity. The virus productivity spread over a wide range of more than 300-fold among different clones, which revealed large variations in their ability to produce progeny viruses. The high and low producer as well as parent cell population were expanded to explore the intracellular virus propagation process, and the expression levels of all the annotated genes were quantified across the different subclones using RNA-seq. The RT-qPCR results showed that the influenza virus RNA synthesis and virus release differed dramatically among subclones during a synchronized single-cycle infection. Pathway analysis performed on the genes indicated that most of the genes are not differentially expressed, but a few key cellular metabolic pathways are differentially expressed among the subclones, especially the genes related to the virus infection, replication and release. These results spurs further hypothesis to improve our mechanistic understanding of cell line stability and virus propagation process, which will have significant impact on rationalizing cell line development of viral vaccine producing mammalian cells.

AN OUTBREED MOUSE MODEL OF YELLOW FEVER FOR STUDY OF PATHOGENESIS AND DEVELOPMENT OF VACCINES AND THERAPEUTICS

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Key Words: Yellow Fever – Animal model – LD_{50} – Vaccine

Yellow fever (YF) is a mosquito-borne viral disease that is endemic in several African and South American countries. YF virus (YFV) causes subclinical infections with mild and non-specific symptoms, to severe, potentially lethal illness with jaundice, hemorrhage, and renal failure. Despite the existence of safe and efficient vaccines, epidemics continue to occur, mostly in Africa where the burden of YF is estimated to represent 1.7 million annual cases and up to 80,000 deaths per year. Moreover, emergence of YF has been reported in new, previously unaffected areas, because of the introduction of the mosquito vectors into these lands. There is no effective therapy against YFV infection but licensed vaccines are available, which are derivatives of a live attenuated strain that was first developed in 1937. These vaccines are currently being used in vaccination program in endemic countries and for travelers visiting these regions. They provide a long-lasting immunity against all the known genotypes of YFV. Although very rare, there are reports of serious adverse effects associated with these vaccines. One major drawback of YF vaccines is their preparation that is based on culture on embryonated eggs, a fastidious and lengthy process that limits the capacity to produce high volumes of stocks needed to respond to recurring epidemics and to prepare for a potential major outbreak. An effective therapy and new types of vaccine that can support rapid scale up is needed for efficient management of YF in the future. The best available animal models to enable these endeavors are currently non-human primates (NHP) in which YF cause a disease similar to human infection of YF. However, the cost NHP studies is a limit to preclinical studies, in particular in the most affected areas of the globe. There are a few mouse models of YF. However, these models consist of genetically-deficient rodents that are not best suited to replicate the disease and to accurately evaluate new vaccines or therapies. We are developing mouse model of YFV infection based on the Swiss Webster outbreed strain. We have tested several epidemics isolates and identified strains that, when administrated by the intraperitoneal route, caused a fulgurant infection leading to death within a week. Death was associated with viral replication in several organs including brain and liver. Interestingly, these YFV strains are lethal only when prepared from mouse organs and not when cultured on cell lines. We used this model to test the efficacy of the 17D YFV vaccine strain in protecting mice against lethal challenge showing that the model can be used to evaluate new YF vaccines and therapies.

DEVELOPMENT OF STABILIZING FORMULATIONS OF A TRIVALENT INACTIVATED POLIOVIRUS VACCINE IN A DRIED STATE FOR DELIVERY IN THE NANOPATCH™ MICROPROJECTION ARRAY

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Key Words: Poliovirus, vaccine, formulation, stability, Nanopatch™

The worldwide switch to inactivated polio vaccines (IPV) is a key component of the overall strategy to achieve and maintain global polio eradication. To this end, new IPV vaccine delivery systems may enhance patient convenience and compliance. In this work, we examine Nanopatch™ (a solid, polymer micro-projection array) which offers potential advantages over standard needle/syringe administration including intradermal delivery and reduced antigen doses. Using trivalent IPV (tIPV) and a purpose-built evaporative dry-down system, candidate tIPV formulations were developed to stabilize tIPV during the drying process and upon storage. Identifying conditions to minimize tIPV potency losses during rehydration and potency testing was a critical first step. Various classes and types of pharmaceutical excipients (~50 total) were then evaluated to mitigate potency losses (measured through D-antigen ELISAs for IPV1, IPV2, and IPV3) during drying and storage. Various concentrations and combinations of stabilizing additives were optimized in terms of tIPV potency retention, and two candidate tIPV formulations containing a cyclodextrin and a reducing agent (e.g., glutathione), maintained ≥80% D-antigen potency during drying and subsequent storage for 4 weeks at 4˚C, and ≥60% potency for 3 weeks at room temperature with the majority of losses occurring within the first day of storage.

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EFFICIENT INFLUENZA VACCINE MANUFACTURING: SINGLE MDCK SUSPENSION CELLS IN CHEMICALLY DEFINED MEDIUM

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Key Words: MDCK suspension cells, influenza virus, chemically defined medium, process optimization, scale-up

Facing the constant global high demand for influenza vaccines, improving production capacity is most important. For influenza vaccine production, cell culture-based processes have advantages regarding flexibility, efficiency, and safety in comparison with the traditional egg-based processes. To avoid problems related to microcarrier-based approaches and serum containing media, growth of suspension cells in chemically-defined media is favoured. In addition, such a process has advantages regarding the improvement of virus titers, the scale-up of the production process, and overall productivity in up- and downstream processing.

In this study, a previously developed MDCK suspension cell line [1] was cultivated in an in-house chemically defined medium to evaluate cell growth and virus production. For the purpose of process intensification, virus adaptation and infection strategies were investigated to achieve high cell densities and to maximize virus titers. Therefore, an adapted influenza virus strain (A/PR/8/34 H1N1 RK1) was generated by a series of virus passages with low multiplicity of infection (MOI). Virus infections were carried out by supplementing 100% of fresh medium, infecting cells with a MOI of 10^{-3}, and with trypsin addition at 72 h of cell cultivations in shake flasks and bioreactors. For scale-up, MDCK cells were cultivated in a DASGIP bioreactor system, optimizing stirring speed, time of infection, pH and DO levels both for cell growth and virus infection. Cell count, viability, main extracellular metabolites, and virus titers were measured to compare productivity between shake flasks and bioreactors.

In batch culture (shake flasks and bioreactors), single MDCK cells were grown to maximum cell densities of 1.2 $x10^7$ cells/ml with cell viabilities exceeding 98% at high cell specific growth rates of 0.036 h^{-1}. Virus adaptation to the MDCK suspension cell line led to a fast infection and stable virus titers over time. Regarding process optimization, optimal pH (cell growth: 7.00, infection: 7.20), DO (40%) and agitation speed (80 rpm) were chosen for influenza A virus production in three parallel bioreactors. Cell densities of 1.0 $x10^7$ cells/ml were achieved at time of infection (72 h) before performing a dilution step. Post infection, similar virus infection dynamics were observed in shake flasks and bioreactors. For both cultivation systems maximal HA titers of 3.6 $\log_{10}$(HAU/100µl) were achieved without reduction of cell-specific virus titer (12,000 virions/cell).

Overall, a highly efficient and scalable upstream process was realized by cultivation of MDCK suspension cells as single cells in chemically defined medium. This is a strong basis for a promising application in large-scale influenza vaccine manufacturing and potential process intensification towards high cell density virus production.

[1] Huang D. et al., PloS One 10 (2015): e0141686. doi: 10.1371/journal.pone.0141686

ANALYTICAL CHARACTERIZATION AND FORMULATION ASSESSMENT OF MODEL SECRETORY-IMMUNOGLOBULIN-A (sIgAs) FOR THEIR POTENTIAL USE AS LOW COST, ORALLY DELIVERED sIgAs

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Key Words: ETEC; sIgA; formulation development; physiochemical method, oral delivery

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of bacterial diarrheal disease in developing countries, especially among children and infants. ETEC is estimated to cause 280-400 million diarrheal episodes per year in children <5 years of age, resulting in 300,000 to 500,000 deaths.[1] Despite the need for a vaccine, there are currently no licensed vaccines against ETEC. Alternatively, passive immunization by oral delivery of pathogen-specific immunoglobulins is another promising approach to provide “instant” protection against ETEC. The potential advantages of oral delivery are reduced cost, simplicity of administration and localized treatment within the GI tract. Secretory IgA (sIgA) is of particular interest because it is naturally found in the mucosal surfaces within the GI tract, relatively more resistant to proteolysis by digestive enzymes (vs. IgGs), and can protect against enteric bacteria by directly neutralizing virulence factors.[2] One major challenge of this approach is the instability of protein molecules during oral delivery (in the digestive tract) as well as during long-term storage (in various formulations). In this study, two proteins, sIgA1 and sIgA2 against heat labile toxin (LT, one of the major virulence factors of ETEC), were used as model sIgA molecules for developing analytical techniques and assessing stability (physicochemical as well as *in vitro* binding) under various conditions. A combination of biochemical and biophysical methods were employed to comprehensively characterize the sIgA1 and sIgA2 model proteins including primary structure, post translational modifications (i.e., N-linked glycans), size, apparent solubility, higher order structure and conformational stability as well as *in vitro* antigen binding. Using these characterization and stability indicating methods, we are monitoring the stability of these two model sIgAs both in an *in vitro* digestion model (to mimic *in vivo* degradation conditions), and during accelerated stability studies (to assess storage stability). Our goal is to use the information gained by these aforementioned methods and stability studies to design stable, low-cost liquid formulations for oral delivery of sIgAs in the developing world.

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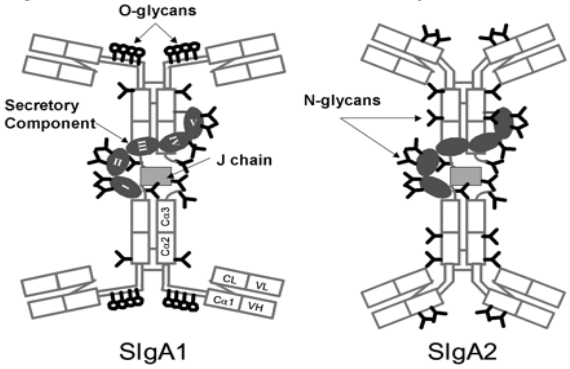

Figure 1: Structure of sIgA [3]

STERIC EXCLUSION CHROMATOGRAPHY FOR THE PURIFICATION OF RECOMBINANT BACULOVIRUS

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Key Words: downstream processing, virus purification, membrane technology, polyethylene glycol, insect cells.

Steric exclusion chromatography (SXC) has already proven to be a valuable tool in the purification of proteins and virus particles. An important benefit of the method is the fast and simple procedure at mild chromatography conditions as no harsh binding and elution buffers are needed. The sample is initially mixed with a polyethylene glycol (PEG) containing buffer of choice. The steric exclusion of a macromolecule from the polyethylene glycol and the stationary phase allows a selective retention of the product, depending, among others, mainly on its size as well as on the molecular weight and concentration of the PEG. Here, SXC was set up in order that smaller process contaminants, i.e. host cell proteins and DNA, did not bind to the stationary phase, in contrast to the targeted larger virus particles. These were subsequently eluted reducing the PEG concentration in the mobile phase. Regenerated cellulose was used as stationary phase to purify VSV-G pseudotyped *Ac*MNPV baculoviruses derived from *Spodoptera frugiperda* cells (Sf9 cells) by SXC. The purified virus particles are used as gene transfer tools for human mesenchymal stroma cells. For this purpose, the baculovirus was clarified prior to the SXC by sequential centrifugation (4700 g_{max}). The SXC conditions were optimized in terms of yield and purity by a design of experiment approach considering the PEG molecular weight, its concentration and the ionic strength of the elution buffer as critical process parameters. Within the design space virus recovery was ≥70%. Without further nuclease treatment the depletion of double-stranded DNA was >90% and the amount of host cell proteins were reduced >90% in the virus fraction.

In conclusion, SXC can drastically reduce the process development in terms of time and equipment requirements for the purification of recombinant baculoviruses, as well as for the achieved purity which is superior over classical methods.

PRODUCTION AND PURIFICATION OF Zika VIRUS FOR AN INACTIVATED VIRUS VACCINE CANDIDATE

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Key Words: Inactivated Zika vaccine

Zika arbovirus is the most recent causative agent of an unattended emerging viral disease. Previously restricted to the African continent, Zika has spread rapidly during the last five years, reaching Asia and America. The emergence of Zika in Brazil revealed that pregnant women is a particular at-risk population due to the possibility of the infection during pregnancy causing congenital Zika syndrome, which in the worst cases is evidenced by severe microcephaly in neonates. Instituto Butantan as a public vaccine producer started studies for the development of an inactivated Zika vaccine as soon as the first birth defects cases came to knowledge. The first strategy chosen for Zika production was based on the production process already established for dengue vaccine. However, in opposition of what was believed at the beginning of the Zika outbreak, this virus has some differential characteristics when compared to Dengue viruses. Mainly due to the lytic behavior of Zika infection, which is not present in Dengue infection, a new process was developed to propagate and purify Zika virions. In order to establish the best culture conditions, Vero cells were seeded in different cell concentrations and culture media, in several flask sizes and types, infected with a range of Zika virus comprising MOI from 0.01 to 0.11, in kinetic studies with or without medium exchange. These studies were responsible for reaching PFU titers above 1E+07 PFU/mL in just 72 h of process with consistent reproducibility in production levels. For purification, harvested Zika was submitted to sucrose gradient ultracentrifugation or to two chromatography steps, reaching the required level of purity regarding host cell protein (< 100 ng/mg) and residual DNA (< 100 pg/dose). Zika vaccine was finally established in more than one formulation, after efficient inactivation with betapropiolactone. Inactivation was carefully evaluated by performing multiple passages of the inactivated material in C636 cells followed by a plaque assay. This work focused not only on generating a proof-of-concept of the immunization with inactivated Zika, but also on the development of scalable process aiming the establishment of a technology ready to enter the next phases of the vaccine development. This project has been funded in part with Federal funds from the U.S. Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority, under Grant No. IDSEP130015. Supported by WHO, Butantan Institute and BARDA.

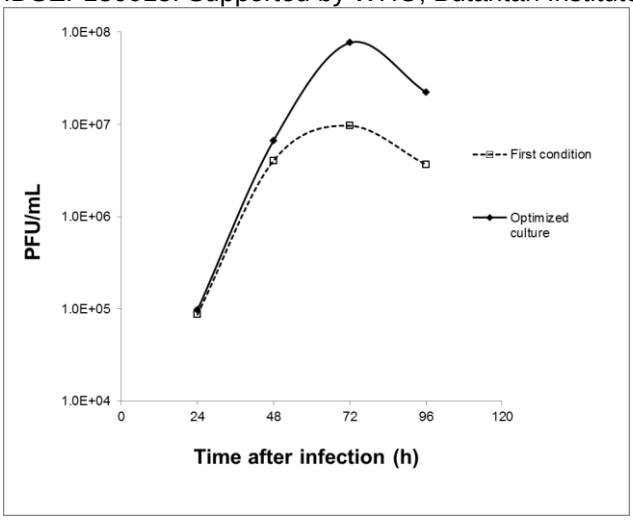

Figure: Zika production kinetic profile. *Zika virus (Strain BeH815744) was inoculated in Vero cells at several conditions in order to establish the best production parameters. The optimization studies resulted in an increase of the mean virus titer from 9.7 E+06 PFU/mL to 7.7 E+07 PFU/mL.*

CO-FORMULATION OF BROADLY NEUTRALIZING ANTIBODIES 3BNC117 AND PGT121: ANALYTICAL CHALLENGES DURING PRE-FORMULATION CHARACTERIZATION AND STORAGE STABILITY STUDIES*

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Key Words: Broadly neutralizing antibodies, Co-formulation, Stability, Protein aggregation

In this study, we investigated analytical challenges associated with the formulation of two broadly neutralizing anti-HIV monoclonal antibodies (bnAbs), 3BNC117 and PGT121, both separately at 100 mg/mL and together at 50 mg/mL each. The bnAb formulations were characterized for relative solubility and conformational stability followed by accelerated and real-time stability studies. While the bnAbs were stable during 4°C storage, incubation at 40°C differentiated their stability profiles. Specific concentration dependent aggregation rates at 30°C and 40°C were measured by size exclusion chromatography for the individual bnAbs with the mixture showing intermediate behavior. Interestingly, while the relative ratio of the two bnAbs remained constant at 4°C, the ratio of 3BNC117 to PGT121 increased in the dimer that formed during storage at 40°C. A mass spectrometry based multi-attribute method (MAM), identified and quantified differences in modifications of the Fab regions for each bnAb within the mixture including clipping, oxidation, deamidation and isomerization sites. Each bnAb showed slight differences in the levels and sites of lysine residue glycations. Together, these data demonstrate the ability to differentiate degradation products from individual antibodies within the bnAb mixture, and that degradation rates are influenced not only by the individual bnAb concentrations but also by the mixture concentration.

*Submitted for publication