# METABOLISM OF AVIAN DESIGNER CELLS DURING INFLUENZA AND MVA PRODUCTION

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As alternatives to well-established lines such as Vero or BHK cultures new designer cells are under consideration for vaccine production. A very successful example for a designer cell is Crucells' human PER.C6 currently either used or evaluated for production of vaccines against Ebola, malaria, West Nile disease and influenza. ProBioGen AG has created avian cells (AGE1.CR and AGE1.CR.pIX) from duck retina using adenovirus type 5 E1 and pIX genes as potential candidates for vaccine production. These avian cells grow in suspension in serum free (SFM) or protein free medium (PFM) and can be productively infected with different influenza strains. Typically, modified vaccinia virus Ankara (MVA) is produced in chicken embryo fibroblasts (CEF), as apart from BHK cells not many other cells are able to replicate MVA. In both avian cells (CR and CR.pIX) MVA can also be produced.

When evaluating new cells for vaccine production metabolic data together with cell density and virus titers need to be characterized for different cultivation conditions such as media, cultivation vessels or infection conditions to identify optimal settings. Here, we present data for cultivation of CR and CR.pIX cells in roller bottles and small scale bioreactors (STR, wave). Surprisingly, pIX appears to impact glutamate and pyruvate metabolism of CR cells. Differences between the two cell lines in metabolism (changes in consumption or release of glucose, lactate, glutamine, ammonia, glutamate and other amino acids) together with cell density in different media will be discussed. Virus replication and yields will be compared to adherent Vero and MDCK cells (for influenza) as well as BHK suspension cells (for MVA). Furthermore, various influenza virus strains (influenza A (H1N1, H3N2), influenza B) will be compared and glycosylation fingerprints of the HA proteins will be shown.

# CAPTURING OF CELL CULTURE DERIVED INFLUENZA VIRUSES BY SULPHATED CELLULOSE MEMBRANES – A PROMISING PSEUDO-AFFINITY METHOD FOR INFLUENZA VACCINE PRODUCTION

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Influenza is a global disease causing several million infections in humans every year. One of the most effective methods in controlling seasonal influenza epidemics is prophylactic vaccination, which requires fast, effective and reliable processes to produce large amounts of vaccine doses every year. Traditionally, the production of human influenza vaccines is based on the growth of viruses in embryonated chicken eggs. However, several limitations are associated with this method including low efficiency, limited scalability and potential allergic reactions induced by egg proteins. Hence, mammalian cell culture based vaccine production has been developed requiring new downstream process strategies for virus purification.

The presented study focused on the development of a pseudo-affinity capture step for Madin Darby canine kidney (MDCK) cell culture derived influenza viruses by sulphated cellulose membranes. Extensive purification studies were done using different influenza virus strains including two strains from the season 2007/2008 (A/Wisconsin/67/2005 (subtype H3N2), B/Malaysia/2506/2004) and A/Puerto Rico/8/34 (subtype H1N1). Viral recoveries based on hemagglutination activity (HA), as well as reduction of host cell dsDNA and total protein were directly compared to results obtained with commercially available cation exchange membrane adsorbers and cellufine$^{TM}$ sulphate. With the modified cellulose membranes a higher viral product recovery was achieved than with cellufine$^{TM}$ sulphate and the cation exchange membrane adsorbers, respectively.

Due to the low back pressure and fast binding kinetics allowing significantly higher flow rates, sulfated cellulose membranes are economically favorable over column based media such as cellufine$^{TM}$ sulphate.

Therefore, sulphated cellulose membranes seem to be an attractive tool for industrial downstream processing of influenza virus in vaccine production. They might even have the potential to replace column based matrix cellufine$^{TM}$ sulphate, which is commonly used for virus purification.

**Poster Number 2**

**ANTI-APOPTOTIC ACTION OF ONE PROTEIN ISOLATED FROM LONOMIA OBLIQUA AND THE MITOCHONDRIAL PARTICIPATION**

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Insect cell system to protein expression has been intensely used for the protein production recombinant. However, in some cases, low expression amounts have limited the industrial production of some proteins of interest. Futhermore, apoptosis death has limited the recombinant protein production. Thus being, one of the forms of to increase the cellular productivity would be to inhibit or to attenuate this cellular death. Recently we have demonstrated that the presence of an potent antiapoptotic protein in Lonomia oblique hemolymph showing that hemolymph supplementation can extend the cell culture viability through apoptosis prevention. Has been reported that mitocondria has one important action in the apoptosis control process, being that mitocondrial membrane permeabilization (MMP) can be an important stage in this process. MMP associated m) eΨor not with the loss of the potential electrochemical of the mitocondria (Δ alteration of the matrix is responsible for the intermembran protein release (e.g. cytochrome c, the AIF, etc) of citosol. As result of this release, some protein (caspases) and DNases are activated and the death process one becomes irreversible. We have observed that the addition of Lonomia obliqua hemolymph to the culture lead to a prolongation of the cellular life (3-4 days) allowing a higher recombinant protein production for baculovírus system in Sf-9 cells or human HEK-293 cells ( more than twice in both system). To identifie the protein with this biological effect, hemolimph was fractionated by ionic exchange and gel filtration chromatographies. Apoptosis death was induced by 500 µm of t-BHP or 600 µm of H202. The presence of apoptosis was characterized by FACS, microscopy electronic and agarosis DNA eletroforesis and the potential m) was determined by JC-1, Hoechst 33324Ψelectrochemical of the mitocondria. (Δ and DIOC6(3). Citocrome C was identified in cytosol by one antibody anti cytochrome. Addition of hemolymph to the apoptosis induced culture avoid the m) suggesting that the antiapoptoticΨcell death and the cells showed a high (Δ effect of this protein can be by it's action in mitocondria membrane, avoiding the lost of the membrane permeability and the cytochrome C liberation .

**Poster Number 3**

**INFLUENZA A VIRUS LIKE PARTICLES AS VACCINE: COMPARISON AND EVALUATION OF DIFFERENT STRATEGIES.**

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Virus-like particles are widely used in vaccine development due to their safety, immunogenicity and relative cost-effective production. Production of virus-like particles can be carried out in various expression systems like yeast, insect cells or plants. Expression of influenza proteins in insect cells leads to the formation of virus-like particles which are a promising vaccine candidate against pandemic and inter-pandemic influenza. We generated influenza virus-like particles by expression of influenza A virus derived proteins HA, NA and M1 by the baculovirus expression system. We also generated influenza virus-like particles by expression of all known influenza proteins except the PB1-F2 protein. As influenza virus-like particles are an interesting tool in recombinant antigen presentation we introduced different epitopes, e.g. from *M. tuberculosis* ESAT-6 into a specific site of the HA-ectodomain. In addition we alternatively tested plants to serve as a production system for virus-like particles. We cloned HA, NA and M1 using suitable expression cassettes into the pGreenII vector. Using *A. tumefaciens* for DNA-transfer we examined the transient expression of influenza proteins in N. benthamiana and the possibilities of formation of influenza virus-like particles, expressing HA, NA ad M1. Influenza virus antigens expressed in forage plants propose an interesting vaccine for oral mass immunizations of poultry.

**Poster Number 4**

**RABIES VIRUS GLYCOPROTEIN (RVGP) EXPRESSION IN DROSOPHILA S2 CELLS AND IN BHK-21 CELL INFECTED BY RECOMBINANT SEMLIKI FORREST VIRUS FOR VACCINE PURPOSE**

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Production of proteins for immunization needs post-translational modifications that are critical for antigenicity. Glycosylsation and correct folding are common related processes involved in the generation of a good quality protein. Therefore, eukaryotic hosts are indicated expression systems for the production of post-translation dependent proteins, as they have the appropriated cell machinery to protein maturation. Here we report the utilization of Drosophila melanogaster Schneider 2 (S2) cells and the Semliki Forest Virus (SFV) expression system to produce the rabies virus glycoprotein (RVGP). They represent different systems with advantages of generating high-level expression of functional membrane proteins, to be relatively easy and safe to handle and scalable. We have constructed gene vectors with or without the BiP signal (i) or the selection hygromycin gene (H) where the inserted RVGP gene is under the control of metalothionein (MT) or actin (Ac) promoter. After transfection S2MTiRVGP, S2MTiRVGP-H, S2MTRVGP, S2MTRVGP-H, S2AcRVGP and S2AcRVGP-H cell populations were selected and the expression of RVGP evaluated by FACS, ELISA, western-blotting. The ability of RVGP to produce antibodies was investigated by “in vivo” mouse immunization. The data showed a higher RVGP expression in S2MTRVGP-H cells (52 % of positive cells, 4 micrograms/1E7 cells). Parameters for storage, lysis and concentration of cells expressing the RVGP were studied for productivity evaluation and future purification. A protocol of cell preparation including cell freezing as dry pellet, cell thawing at 4ºC with Tris, NaCl, MgCl2, PMSF and cell lysis with the buffer containing NP-40 was chosen, since it fulfilled requirements of high RVGP detection, easiest and quick frozen and cost saving lysis buffer formulation. Selection of an adherent S2AcGPV2K cell population enabled us to enrich the RVGP expression from 0.5 to 3 micrograms/1E7 cells (30 to 60 % of positive cells). Western blotting analysis showed a 65 KDa RVGP monomeric structure. High levels of antibodies against RVGP were found in immunized mice. The RVGP gene was also cloned into a pSFV expression vector. BHK-21 cells were electroporated with expression and helper RNA vectors and SFV-GPV particles were obtained. These were used to infect BHK-21 cells and the RVGP expression was confirmed by western blotting, ELISA and cell immunofluorescence. These data show several steps of optimization for high RVGP expression in S2 cells, its synthesis in a high degree of quality and the RVGP expression in the SFV system.
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**Poster Number 5**

# TOWARDS A RECOMBINANT VACCINE FOR HEARTWATER

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*Ehrlichia ruminantium* is an obligate intracellular pathogen and, as such, cell mediated immunity plays a key role in the control of bacterial replication and subsequent protection against heartwater. Thus, identification of antigens that preferentially activate T cells to proliferate and secrete IFN-gamma need to be evaluated as vaccine candidates. Previously a cocktail of four open reading frames were identified that induce IFN-gamma and 100% protection after needle challenge but only 20% protection after tick challenge in the field, when administered as a DNA vaccine in sheep. Considering that limited protection was obtained during a field vaccine trial our research is focused to improve this efficacy. Because secreted proteins are reported to be major targets in the specific immune response we hypothesise that they may be potential heartwater vaccine candidates. We selected five ORFs encoding secreted *E. ruminantium* proteins from the Welgevonden stock genome sequence using bioinformatics tools. The corresponding recombinant proteins were expressed in a bacterial expression system and assayed to determine whether they induce recall cellular immune responses *in vitro*. Only four recombinant proteins could be expressed. Significant proliferative responses were evident for 3/4 recombinant proteins. IFN-gamma production was determined using an ELISPOT assay and 3/4 recombinant proteins induced IFN-gamma production. Each recombinant protein had its own optimum concentration for inducing immune responses and the responses differed between animals. Thus these three proteins that induce proliferation and IFN-gamma production may be important in protection against heartwater and will be tested in future vaccine studies.

# CRYO-ELECTRON MICROSCOPY AS A TOOL FOR IMAGING, CHARACTERIZATION AND STRUCTURAL ANALYSIS OF BIOLOGICAL SOLUTIONS

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Cryo-transmission electron microscopy (cryoEM) provides a method for directly observing macromolecules in their native hydrated state, avoiding the artifacts associated with alternative preparation methods like negative staining. While cryoEM can provide invaluable data for sizing and structural analysis, this technology has not in the past been suitable as a standard characterization method. However, recent advances in microscope control and automation have streamlined cryoEM data acquisition and analysis and, in conjunction with an integrated database, the method now provides a controlled and routine process suitable for biopharmaceutical and biotechnology applications. We will provide an overview of cryoEM and demonstrate the advantages of the technique using specific examples. In particular, we will describe a collaboration with Merck & Co., on the characterization of a quadrivalent human papillomavirus VLP vaccine (Gardasil™). Automated cryoEM methods were used to accurately determine bulk size distributions of the VLPs assembled from four different serotypes. Particles of a specific size range were then selected and used to reconstruct three dimensional (3D) electron density maps of the VLPs. CyoEM imaging provides direct visualization of the particles in solution and the 3D reconstruction provides a comparison with theoretical and experimental models of the structure. CryoEM also provides very specific and accurate size analysis of the sample population and the ability to visualize aggregates within the sample. These data can be used to calibrate other bulk sample measurements, for example light-scattering.

# INSECT CELLS AS AN EFFICIENT PLATFORM FOR THE PRODUCTION OF AAV-BASED VACCINES

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Given the ability to efficiently transduce human dendritic cells, adeno-associated viruses are promising vaccine candidates. It is known, however, that the full utility of this class of vector has been hampered by the difficulty in producing high-titer AAV stocks. Since the ability to produce AAV vectors in insect cells was first described in 2002, a deeper understanding of the system has advanced the state of the art towards realizing the full potential of this system. Three baculovectors are used: BacRep, for the replication proteins; BacCap, for the structural proteins; and BacITR, for the AAV vector genome. The baculovectors were designed such that the Rep78 gene, under the control of a ΔIE1 promoter, is expressed earlier and to a lesser extent than other AAV proteins in the system, whose expressions are driven by polyhedrin promoters. The flexibility that co-infection systems generally offer is restricted with this design, and the relative time of infection impacts the quantity of AAV vector produced. A delay in the addition of BacRep by 12 hours results in a dramatic loss of AAV vector production. Both quantity and the time of addition of the BacITR affect AAV vector production the least, supporting that the availability of the "template" is not the bottleneck of the process. Increasing culture temperature increases the early production of Rep78 and Rep52, concomitant to a significant increase in AAV vectors produced. Given this understanding we have investigated production strategies at increased cell densities, which ensure the cells receive BacRep. Although specific cell productions is maintained up to ~10x10^6 cells/ml using a medium renewal strategy, a fed-batch strategy, more amenable to the production at large scale allowed us to maintain the specific production up to ~8x10^6 cells/ml (650 ETU/cell). The current approach is based on combining an asynchronous/synchronous infection. This alternative strategy allows the propagation of one of the viruses (BacITR) from a low MOI. The other viruses are then added at a point when the primary virus has been amplified to the extent of being able to infect the complete culture. Given an initial MOI of 0.1 pfu/cell, the optimal time of addition of the other viruses was 27 hpi, which gave an increase in volumetric production of 237% compared to cultures co-infected with all three baculovectors at MOIs of 3 each. These results provide more incentive to use the baculovirus/insect cell derived AAV vectors for large clinical investigations.

# ENGINEERING OF AN *E. COLI* HOST FOR PRODUCTION OF PLASMID BIOPHARMACEUTICALS

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In recent years, gene therapies and DNA vaccines have captivated the scientific and medical communities with their potential to yield new, specific, and safe treatments for many devastating diseases. Plasmid DNA-based therapies represent a substantial portion of current research efforts. As these therapies move into clinical trials and toward FDA approval, the demand for pure, high-quality plasmid DNA will increase. Current plasmid production processes almost exclusively use common laboratory host strains of *Escherichia coli* K-12 like DH5alpha. These strains have been designed for cloning or recombinant protein production, and as a result have been heavily mutagenized. This mutagenesis could potentially affect cell survival and metabolite utilization when compared to wild type cells. Successful large-scale manufacture of plasmid DNA requires robust host cells that yield high biomass and minimize the production of impurities.

This work will engineer a wild-type strain of *E. coli* for enhanced plasmid DNA yield using systematic gene knockouts. The *recA* and *endA* genes will be mutated first, as disruption of these genes is known to improve plasmid stability and maintenance. With most of its natural survival mechanisms still intact, we predict that this new strain will be better equipped to withstand the burdens of high-density cell culture and overproduction of plasmid DNA. The plasmid production capacity of the engineered strain will be compared to that of both wild-type *E. coli* and a common laboratory strain. Preliminary results comparing the plasmid production of wild-type and laboratory strains suggest that strains with a genotype similar to wild-type may yield higher concentrations of plasmid DNA.

**Poster Number 9**

# RAPID DEPLOYMENT PLASMID PRODUCTION: COMBINING INDUCIBLE HIGH YIELD FERMENTATION PROCESS WITH NOVEL AUTOLYTIC PLASMID DNA PURIFICATION

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DNA vaccines and gene medicines, derived from bacterial plasmids, are emerging as an important new class of pharmaceuticals. They may allow the manufacturer to bypass years of development for the production of efficacious vaccines, and literally create new vaccine entities and mass produce vaccines in 2-3 weeks for rapid deployment against new biological agents. However, the challenges of producing plasmid DNA at an industrial scale are well known: low bioreactor yields, scaling up alkaline lysis, clearance of host RNA and chromosomal DNA, and avoiding the use of animal sourced products. This limits their utility to meet cost and capacity needs for existing plasmid applications, or to rapidly produce kilograms of plasmid DNA for pandemic vaccination.

The development of an inducible fed-batch fermentation process that dramatically increases volumetric yield and specific plasmid yield, while maintaining or enhancing plasmid integrity has been the first achievement toward these goals. This inducible process utilizes commercially available media that we designed specifically for plasmid production. The process consists of an initial biomass accumulation phase, followed by a plasmid accumulation phase. The plasmid is stably maintained at low levels during a period of nutrient restricted growth and reduced temperature (30°C), and then the temperature is increased (37-42°C) to induce plasmid amplification. Typically, the specific plasmid yield increases over a period of up to 15 hours following temperature up-shift. Volumetric yields exceeding 2.1 g plasmid DNA/L have been achieved with this process, and this process has been successfully scaled up for GMP production.

To address downstream purification challenges, we have successfully demonstrated feasibility of a cost effective and simple purification process that eliminates costly alkaline or heat lysis steps and the associated toxic waste streams. We developed autolytic *E. coli* host strains that express endolysin after the temperature increase in the fermentation. In the autolytic purification process, lysis is performed at moderate temperatures and without the addition of lysozyme.

The overall result is a rapid deployment plasmid production system linking autolytic plasmid purification to our existing fermentation platform to facilitate immediate production of a variety of plasmid DNAs for pandemic applications. The major benefits of this production platform are speed, safety, simplicity, scalability and transferability to existing manufacturing facilities in developed and emerging countries.

**MODIFIED E. COLI B, A SUPERIOR PRODUCER OF PLASMID DNA COMPARED WITH E. COLI K.**

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Plasmid DNA is an emerging experimental vaccine produced in E. coli that was initially targeted for viral diseases. Unlike traditional protein vaccines when average dose is micrograms, the average dose of pDNA is milligrams; therefore, production yields are critically important for the future development of this vaccine. The E. coli strains currently used for pDNA production, JM109 and DH5a, are both suitable for production of stable pDNA due to the deletion of recA and endA, but at the same time, these two E. coli K strains are sensitive to growth conditions such as high glucose concentration. E. coli BL21 is less sensitive to growth conditions than E. coli JM109 or DH5a, it grows to higher densities and due to its active glyoxylate shunt and anaplerotic pathways is not sensitive to high glucose concentration. This strain is used intensively for recombinant protein production but not for pDNA production because of its inability to produce stable pDNA. To adapt E. coli BL 21 for stable pDNA production, the strain was mutated by deleting both recA and endA, and proper growth and production strategy was developed. Volumetric production values exceeding 2 grams per liter were obtained using glucose as carbon source. The produced plasmid, which was constructed for HIV clinical purpose, was found to be identical in its properties to the plasmid currently produced by E. coli DH5a.

# IMMUNOGENIC DISPLAY OF DIVERSE PEPTIDES ON VIRUS-LIKE PARTICLES OF RNA PHAGE MS2

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The high immunogenicity of peptides displayed in dense repetitive arrays on virus-like particles makes recombinant VLPs promising vaccine carriers. We describe a platform for vaccine development based on the VLPs of RNA bacteriophage MS2. It serves for the engineered display of specific peptide sequences, but should also allow the construction of random peptide libraries from which specific binding activities can be recovered by affinity selection. Peptides representing the V3 loop of HIV gp120 and the ECL2 loop of the HIV coreceptor, CCR5, were inserted into a surface loop of MS2 coat protein. Both insertions disrupted coat protein folding and VLP assembly, but these defects were efficiently suppressed by genetically fusing coat protein's two identical polypeptides into a single-chain dimer. The resulting VLPs displayed the V3 and ECL2 peptides on their surfaces where they showed the potent immunogenicity that is the hallmark of VLP-displayed antigens. Experiments with random-sequence peptide libraries show the single-chain dimer to be highly tolerant of 6-, 8- and 10-amino acid insertions. Not only do MS2 VLPs support the display of a wide diversity of peptides in a highly immunogenic format, but they also encapsidate the mRNAs that direct their synthesis, thus establishing the genotype/phenotype linkage necessary for recovery of affinity selected sequences. The single-chain MS2 VLP therefore unites in a single structural platform the selective power of phage display with the high immunogenicity of VLPs.

# NOVEL TECHNIQUES FOR CHARACTERIZATION OF DOUBLE AND TRIPLE-LAYERED ROTAVIRUS-LIKE PARTICLES

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Rotavirus-like particles (RLPs), candidate vaccines against Rotavirus disease, can be produced in insect cells by co-infection of baculoviruses coding for VP2 and VP6 for double-layered RLP 2/6 as well as for VP2, VP6 and VP7 in triple-layered RLP 2/6/7. Analytical methods currently used for virus-like particles (VLPs) characterization include SEC-HPLC, TEM, SDS-PAGE and Western Blot. However, these methodologies are either time-consuming, have little sensitivity, and thus are semi-quantitative or involve a significant amount of sample. Moreover, they do not give information on the particle's stoichiometry and assembly, which are important issues for improving VLP yield.

In this work novel techniques were applied for RLP 2/6 and RLP 2/6/7 characterization. SDS-capillary gel electrophoresis (SDS-CGE) was successfully used for quantitative and qualitative characterization of RLPs in terms of detecting the presence and relative proportion of VPs for quality control purposes. The method demonstrated to be accurate for apparent molecular weight estimation and allowed evaluation of VP7's degree of glycosylation. Its precision is sufficient for detection of VP6/VP7 ratio differences between different samples as well as for protein quantification.

Another extremely useful technique to characterize VLPs is dynamic light scattering (DLS), which enables measurement of zeta potential, size and molecular weight. The average diameters of RLP 2/6 and RLP 2/6/7 were 50 and 80 nm, respectively. Several physical-chemical conditions (pH, ionic strength, temperature and chelating agent concentration) were used for size and zeta potential measurement. Besides giving valuable information on RLPs stability and disassembly, DLS also enabled the plot of a titration curve for pI calculation, which was 2.7 (RLP 2/6/7).

Using both methods, stoichiometry, assembly and disassembly kinetics of RLPs are possible to evaluate and quantify.

**HOST STRAIN INFLUENCES ON SUPERCOILED PLASMID DNA PRODUCTION IN *E. COLI*; IMPLICATIONS FOR EFFICIENT DESIGN OF LARGE SCALE PROCESSES.**

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With many gene based vaccines/therapies in early development, there is a growing need for quick and effective methods to obtain relatively large amounts of supercoiled plasmid DNA. Whilst much effort has been directed towards the downstream processing, there is much room for improvements at the earlier stages of production namely the cell host/plasmid selection where the choice of the *E. coli* host strain used to propagate the product containing-plasmid may have sizeable influence on the quality and quantity of the purified DNA. This would influence both production as well as the downstream operations.

In this investigation, a systematic approach has been conducted to carry out experiments with 17 *E. coli* strains and 3 plasmids to determine the influence of the host strain on the quantity and quality of DNA obtained, particularly supercoiled DNA. The strains selected range from well known commercial strains to the lesser used ones from the early era of microbiology. The data obtained from this investigation can provide an effective tool in selecting a host strain for a specific plasmid-based product, potentially reducing manufacturing costs by aiding the productivity of the system and improving the downstream processing stages. The study shows that the choice of host strain strongly affects the total yield of plasmid DNA. In cultures containing two relatively similar-sized plasmids and with similar high copy numbers some strains gave a higher yield with one plasmid (e.g. MG1655 with gWiz) and not with the other (e.g. MG1655 with pSVbeta). On the other hand, there were strains that grew well and provided consistent high yields of plasmid DNA and also SC-DNA for both these plasmid cultures and these were DH5alpha, BL21 DE3, TG1 and HB101 at 10ml and 100ml scales. All tested strains propagating a large plasmid (20kb) generally produced lower plasmid DNA yields than obtained from cultures producing smaller plasmids (5-7 kb). The results also show that the presence of certain host strain mutations previously reported to aid the quality of plasmid DNA such as *endA* and *recA*, does not always guarantee high supercoiled DNA yield. Higher specific growth rates of some strains did not affect the level of supercoiling but did influence the total DNA plasmid yield, making an important finding in the design of fermentation strategy.

**Poster Number 14**

# INSIGHTS INTO THE EFFECTS OF CULTURE MEDIA AND METABOLITES ON ADENOVIRUS PRODUCTION

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Adenoviruses have transitioned from tools for gene replacement therapy to bona fide vaccine delivery vehicles. They are attractive vaccine vectors as they induce both innate and adaptive immune responses in mammalian hosts. Currently, adenovirus vectors are being tested as subunit vaccine systems for numerous infectious agents ranging from malaria to HIV-1. To meet the growing market requirements and reduce the final cost of vaccine production, large amounts of adenoviral vectors need to be generated by designing efficient and scaleable processes.

Adenovirus production is currently limited by reduced cell-specific productivity observed at high cell density infection. This so-called “cell density effect” is probably due to nutrient limitations and/or metabolite inhibition. Though various feeding strategies have been explored to overcome this effect, only limited success has been achieved. The exact nature of the limitations remains unknown. To better understand these limitations, we first compared the performances of three culture media (CD 293, Ex-Cell 293 and NSFM13) for growth of 293SF cell (clone of HEK-293) and for production of adenovirus. Ex-Cell 293 promoted the best cell growth, however, the cells grown in this medium produced low virus yields. In contrast, CD 293 did not support 293SF cell growth after few cell passages while it was the best medium for virus production. The nutritional requirements of 293SF cells appear to be different between cell growth and virus production phases.

The cell density effect was prevalent in cultures using the above media, however the decline in the virus production was far more drastic (98%) in NSFM 13 culture than in other two cultures when the cell density at infection was increased from 0.5 to $3x10^6$ cells/mL. The attribution of nutrient limitation and metabolite accumulation to the cell density effect was investigated by supplementing potential limiting nutrients or inhibitory metabolites (lactate and ammonia) to infected cultures. Lactate and ammonia generated during virus production contributed only partially (about 30% decline) to the observed cell density effect, while nutrient limitation was primarily responsible for the drastic decline in the virus production when NSFM 13 was used as production medium. The portion of cell density effect associated with nutrient limitation can be minimized through supplement of nutrients or national medium formulation.

# NOVEL ADENOVIRUS 5 VACCINE DELIVERY PLATFORM WHICH OVERCOMES PRE-EXISTING IMMUNITY TO AD

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Vaccine studies using Adenovirus (Ad) vectors have been hampered due to the barrier of pre-existing immunologic responses against Ad in many cases. This has resulted in a variety of protocols designed to overcome this limitation. In an attempt to avoid the barrier of pre-existing immunity, we have evaluated a novel [E1-, E2b-] Ad serotype 5 (utilizing the E.C7 cell line for viral packaging) vector vaccine to assess its effectiveness as a potential vaccine platform as compared to the currently utilized [E1-] Ad5 based platforms. The new human [E1-, E2b-] Ad5 vector vaccines have several advantages. Most importantly, Ad viral DNA replication is significantly diminished due to the removal of the E2b region. This results in at least a 10,000-fold reduction in the production of Ad late gene products. The decrease in late gene products reduces the potential of Ad encoded viral proteins from impacting host immune responses by decreasing antigenic competition from the Ad genes and enhanced transgene expression resulting in increased immunologic stimuli.

The present studies were designed to construct and test the effectiveness of an HIV-1 vaccine platform based on the new [E1-, E2b-] Ad vector platform in vivo. [E1-] Ad and [E1-, E2b-] Ad vector vaccines having Gag, Pol or Nef inserts were used. Ad vaccines were tested for their potential to induce HIV antigen memory CMI responses and antibody in a prime and boost protocol in Ad-naïve and Ad-immune mice. We report that the Ad5 vectors are equally immunogenic in Ad naïve mice. [E1-, E2b-] Ad vector vaccines had statistically greater immunogenic propensity in Ad immunized mice, as compared to [E1-] Ad vector vaccines. Thus, the [E1-, E2b-] Ad vector vaccines were successfully employed in a prime-boost protocol in both Ad naïve and Ad immune mice, whereas [E1-] Ad vectors were only effective in Ad naïve. The [E1-, E2b-] Ad vaccines induced significantly greater numbers of antigen specific CD8+ T-cells and CD4+ T-cells. We hypothesize that the greater number of CD4+ T-cells was a result of higher concentrations of IL-2 induced by [E1-, E2b-] Ad vector vaccines. These results indicate that one can immunize Ad naïve and Ad immune vaccinees using the new [E1-, E2b-] Ad vector vaccine platform in a prime-boost manner.

# FERMENTATION STRATEGIES FOR THE PRODUCTION OF RECOMBINANT PROTEIN ANTIGENS IN E. COLI

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E. coli has been widely used as a host for the production of recombinant proteins. In this presentation, two examples of high cell density recombinant E.coli BLR fermentation will be discussed. In the first study, the pBAD expression system was used since it affords a simple method for regulating protein production to maximize yield. However, the araBAD promoter is catabolite repressed if glucose is present above certain concentrations in the culture medium during induction with arabinose. To more efficiently utilize arabinose, simultaneously feeding arabinose and glucose was investigated. By optimizing the feeding and induction strategies, arabinose usage was reduced significantly, resulting in high cell densities (OD of about 100) and recombinant protein production of approximately 2 g/L.

In the second study, the T7 expression system (pET vector) was used, which requires IPTG for protein induction. It was discovered that isoleucine was required when chemically defined medium was used during fermentation of E. coli BLR (DE3) host strains. To achieve high cell densities, isoleucine together with glucose was fed during fermentation. Using design of experiments (DOE) methodology, induction OD, IPTG concentration, and post-induction feed rates were assessed. Induction OD was the only significant factor in determining maximal protein production. Final OD of about 100 with recombinant protein titers of approximately 2 g/L were achieved after optimization.

**CRITICAL PROCESS PARAMETER TO CONTROL PRODUCTIVITY IN HELPER-DEPENDENT ADENOVIRAL VECTOR PRODUCTION**

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Helper-dependent adenoviral vectors (HDV), devoid of viral coding genes, are attractive vehicles for vaccination. They do not trigger the adaptive immune response, a major drawback in vaccine efficacy. Despite an increasing demand for this vector, production is still non-efficient as a result of an empirical process. A HEK293 cell line is usually co-infected with HDV and a first generation vector called Helper Virus (HV). A recombinase system is generally used to restrict HV packaging. In an attempt to improve HDV productivity while decreasing HV contamination, strategies to manipulate the stoechiometry of HDV components were evaluated. The multiplicity of infection (MOI) of both viruses was a simple parameter not only to improve HDV productivity but also to limit HV contamination. Using a quantitative PCR assay to compare HDV and HV titers, we found that HDV titer was dependent on the co-infection ratio whereas HV titer was only related to the MOI of HV. Consequently, HV contamination could be dramatically high when high MOI of HV is used (up to 60 times more HV than HDV with MOI HV 10). At optimal co-infection conditions, $2x10^8$ infectious HDV/mL were obtained with only 1% HV contamination. Delaying HV infection did not result in higher HDV and/or lower HV titers. Together with the use of an efficient recombinase system, co-infection with optimal amount of viruses guarantees the quality of HDV stock before purification.

**Poster Number 18**

**PRODUCTION OF YELLOW FEVER VIRUS IN VERO CELLS GROWN IN SERUM-FREE MEDIUM**

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The attenuated vaccine against yellow fever (YF) virus produced in embryonated eggs at FIOCRUZ (Brazil) has been available for decades. This vaccine has been used for human immunization with an excellent history of efficacy and safety. However, in the latest years, the occurrence of adverse events associated with the 17D and 17DD substrains indicated the need for developing technologies for the production of an inactivated vaccine. With this aim, the use of continuous cell lines for production of the antigen offers advantages over viruses grown in embryonated eggs. A major advantage is the production of virus in large scale under controlled conditions (pH, temperature, dissolved $O_2$).

Vero is a continuous, adherent cell line, which has been recommended by the World Health Organization for the production of human vaccines. Commercial processes using this cell line for vaccine production are based on its culture on microcarriers in stirred systems. The aim of this study was the development of an efficient cell culture process and an adequate infection strategy for propagating the yellow fever virus in Vero cells using stirred bioreactors.

Using the best conditions determined for cell propagation and viral infection, employing a serum-free medium, very high virus titers ($10^8$ pfu/mL) were obtained, indicating that the methodology developed in this work is efficient and could be employed in a process for the production of yellow fever virus. Analysis of the antigenic properties of the 17DD virus by an enzyme-linked immunoassay showed no difference between the virus cultivated in serum-containing and in serum-free medium. In addition, SDS-PAGE analysis of the supernatant obtained from infected cell cultures confirmed a drastic reduction in the protein content of samples of the YF virus cultivated in serum-free medium. Thus, the development of a cell culture process for propagating the yellow fever virus in Vero cells grown in serum-free conditions can represent an important step towards the production of a new, inactivated vaccine against yellow fever.

**SCREENING OF DNA VACCINES PROTOTYPES ENCODING ANTIGEN TARGETING SEQUENCES AGAINST SLEEPING SICKNESS**

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Sleeping Sickness is an emergent disease and an important cause of mortality in sub-Saharan Africa. The annual reported number of infected people is 30 to 50 000 but unfortunately this estimative is considered to be the tip of the iceberg as the actual number could be as high as 300 000 cases per year. The current drug regimens are unacceptable due to serious side effects, so the development of new therapeutics, as a vaccine, is urgent.

Ten DNA vaccine prototypes for African Trypanosomosis were constructed encoding ISG and TSA genes from *Trypanosoma brucei* containing different targeting protein sequences to the major processing of antigens pathways: (1) LAMP-1 signal targets the protein to the lysosomes (ISG-LAMP, TSA-LAMP); (2) E1A directs the protein to the ER (E1A-ISG, E1A-TSA) (3) secretion signal promotes the protein secretion (Sc-ISG, Sc-TSA) (4) E1A and LAMP combined (E1A-ISG-LAMP, E1A TSA-LAMP).

CD1 mice were immunised with 50µg of each candidate vaccine and blood samples were taken for the determination of anti-ISG and anti-TSA antibodies by ELISA assays. Animals were sacrificed 28 days post-immunization and axillary and popliteal lymph nodes were taken to perform immunohistochemistry analysis and mRNA extraction for cytokine quantification (IL 2, IL 4, IL 6, IL 10, IFNg and TNFá). ISG-LAMP, TSA LAMP and ISG-E1A presented higher expression levels for most of the cytokines tested. Haematoxylin and eosin histological sections of right popliteal lymph nodes taken from mice immunized with ISG LAMP and E1A-ISG-LAMP have shown the typical characteristics found in activated lymph nodes (such as lymphatic nodules with germinal centres and lymph vessels with lymphocytes). Cell-mediated immunity was also determined through the DTH response measured by the footpad-swelling test. The immunised CD1 mice have received a trypanosome lysate on the right footpad 14 days post immunization (left footpad was injected with saline) and the footpad's thickness was monitored at 24 and 48 hours after injection. At 24h the footpad thickness of mice injected with ISG LAMP was significantly higher than the control.

The study allowed us to select the most promising vaccines (ISG- LAMP, TSA LAMP and E1A-ISG) that will be used in challenge assays to determine its effectiveness on the survival rates of infected mice.

# ON THE DESIGN AND PRODUCTION OF MORE STABLE AND EFFICIENT PLASMID DNA VECTORS

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DNA vaccination has matured to the point where a number of products should be hitting the market in the wake of the first veterinary DNA vaccines approved in 2005. Apart from their potential to prevent and treat infectious and acquired diseases, DNA vaccines offer advantages such as the rapid and generic manufacturing, good stability at high and low temperatures and low cost. The design and large scale production of plasmid DNA (pDNA), the active component of a DNA vaccine, are two key aspects underlying the successful development of a DNA vaccine.

We have developed a patented process based on hydrophobic interaction chromatography (HIC) for the production and purification of pDNA vectors. Comparison of this method with an isolation process based on a glass fiber fleece matrix shows that the purification method used can strongly affect transfection efficiency of cultured CHO cells. Although quality analysis by standard analytical techniques did not detect significant differences between plasmids purified by the two methods and no differences on pDNA copy number in transfected cells were measured, an increase in mRNA copies (2.2-fold) and also in the reporter protein was found in cells transfected with HIC-purified plasmids. Circular dichroism analysis and SDS-PAGE indicate that these differences should be ascribed to trace amounts of *E.coli* proteins which inhibit transcription and/or mRNA maturation and stability.

Nuclease degradation of DNA vaccines after delivery and during trafficking to the nucleus is a major barrier to gene expression and consequently to the elicitation of immune response. Our approach to reduce this problem relies on the construction of pDNA variants that are more resistant to nuclease action. This was achieved by modifying the polyadenylation and origin of replication in a model plasmid backbone. Although those regions contain non-B DNA secondary structures which facilitate the binding of specific proteins, our studies indicate that in the case of the origin of replication, nuclease resistance and transfection efficiency can be improved 2.5-fold and 1.5 fold, respectively, by replacing as few as 7 nucleotides without loss of functionality. In conclusion, the choice of plasmid vector sequences is important, not only for mRNA maturation/stability, but also for pDNA resistance, and should thus be taken into consideration when designing and evaluating pDNA vectors.

Moreover, we have some evidence that the use of plasmids which are more resistant to nucleases is likely to have a positive impact in downstream processing and product shelf stability.

**VP7 AND VP4 GENOTYPING OF BOVINE GROUP A ROTAVIRUS IN MéXICO. TOWARDS THE DEVELOPMENT OF A RECOMBINANT VACCINE.**

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Group A rotavirus is the main cause of neonatal diarrhea in bovines and porcines. Vaccination has proven to be helpful for the prevention of this pathology, which causes important economic losses. Several groups have shown that virus like particles (VLP) inoculated into pregnant animals induce high antibody responses in mammary secretions; calves fed with colostrum or milk from vaccinated animals are protected from diarrhea following rotavirus challenge. Two proteins, VP7 and VP4, on the external viral capsid carry type-specific antigens and are responsible for the dual rotavirus classification into G (glycoprotein) and P (protease sensitive) serotypes/genotypes, respectively. Genotyping has been preferred to serotyping, due to its high sensitivity and use of universal reagents. Over 15 G and 26 P types have been identified to date. Genotyping of group A rotavirus is an essential tool for epidemiological studies of rotaviruses and is also important for vaccination, as it has been shown that protection between genotypes is limited. Therefore, it is necessary to incorporate the predominant genotypes in vaccines for a specific region to confer optimal passive immunity to calves. No previous reports exist on the group A rotavirus genotypes in Mexican bovine cattle. The goal of this study was to determine the genotypes G and P of group A rotavirus detected in fecal samples from calves in Mexico. One hundred twenty-eight fecal samples from calves with diarrhea were collected between 2005 and 2006 from 26 dairy and/or beef cattle herds located in 10 regions of Mexico. Rotavirus group A was confirmed prior to genotyping by a rapid immunoassay for detection of VP6 rotavirus antigen. Genotyping was corroborated by sequence analysis. Rotavirus was identified in 15.6% of liquid stool samples. Calves born from cows vaccinated against G6, P[1] genotype (commercial vaccine) were affected by the G10, P[11] genotype. These results show that protection between genotypes was limited. The genotypes found were: G10, P[11] (67%); G6, P[5] (25%) and G10, P[5] (8%). These results show a clear relationship between genotype and species, as these combinations are commonly found in bovine rotavirus. It was also found that the commercial vaccine containing genotypes G6, P[1] provides partial heterotypic immunity against the G10, P[11] genotype. The genes isolated from the samples have been used for the production of VLPs using the insect cell-baculovirus expression vector system (IC-BEVS), or yeast. This is the first approach towards the development of a recombinant rotavirus veterinary vaccine in México.

# 293 CELLS AN ALTERNATIVE CELL LINE FOR PPRV PRODUCTION

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Peste des Petits Ruminants virus (PPRV) is the causative agent of a highly contagious and fatal disease of sheep and goats and some small wild ruminant species that presents a significant economic impact in African and Asian countries.

Currently the best strategy to control PPR is by vaccination with an attenuated strain produced in Vero cells. However, this cell line is anchorage dependent which can limit the scalability of the production process, as microcarrier technology is required. This work reports on the comparison of 293 cells, which are able to grow in single cell suspension using serum free media, with Vero cells for the production of PPRV. Similar volumetric productivities were achieved for both cell lines, namely $10^6$ $TCID_{50}$/mL, even when using low MOIs like 0.001.

Moreover, being thermal stability a critical issue for vaccine delivery in developing countries, different formulations were tested and the effect of stabilizers, such as sucrose and trehalose were evaluated. The thermal inactivation of PPRV produced with the two cell lines in static conditions showed that virus produced in 293 cells were slightly less stable than those produced in Vero cells. A liquid formulation composed of a Tris buffer supplemented with trehalose was tested permitting a half-life of one month at 4ºC and 21 hours at 37ºC for virus produced in Vero cells.

A good candidate formulation (Tris/trehalose formulation) was obtained for the current PPR vaccine without the need of lyophilization. This constitutes the first report on a PPR candidate vaccine produced in suspension culture with serum free media. Although more studies are necessary to optimize the production and minimize the viral infectivity losses, the results present herein constitute valuable information on the development of a large-scale cell culture process for PPR vaccine.

**Poster Number 23**

# ASSESSMENT OF THE THERMAL STABILITY OF CERVARIX ™

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Cervarix™ is a vaccine protecting against 'high-grade' cervical intraepithelial neoplasia and cervical cancer that are caused by the human papillomavirus (HPV) types 16 and 18. This vaccine is a suspension for injection that contains purified L1 proteins of HPV-16 and HPV-18 assembled as virus-like-proteins (VLPs) combined with the AS04 adjuvant system.

The thermal stability of Cervarix™ was evaluated in three different studies that assessed: (1) the long-term stability which establishes the stability of the vaccine under proposed storage conditions following testing at various time points over the entire targeted shelf life period, (2) the accelerated stability which provides useful support data for establishing the expiration date and reveals the pattern of degradation under extreme storage conditions and (3) whether accidental exposures to conditions other than those proposed for long-term storage (e.g., during transportation) are deleterious to the product (transport test). The thermal stability was evaluated by monitoring the potency, the purity and the quality of the vaccine using physicochemical and immunological methods, such as the *in vitro* and *in vivo* potency.

The long-term stability study showed that the vaccine is stable for the proposed 36-month shelf life when stored at +2°C to +8°C. The accelerated stability study showed that the vaccine is stable after storage for 7 days at 37°C without any pattern of product degradation. The transport test study showed that the vaccine is stable when exposed 7 days at 37°C after 9 months storage at +2°C to +8°C followed by a further storage for 27 months at +2°C to +8°C. Further data showed that Cervarix™ is also stable when exposed 14 days at 25°C after 9 months storage at +2°C to +8°C followed by a further storage of 27 months and when exposed 30 days at 25°C after 11 months storage at +2°C to +8°C.

Through the evaluation of immunological and physico-chemical stability-indicating parameters, Cervarix™ is shown to be stable for 3 years under proposed storage conditions, under accelerated and extreme storage conditions as well as when accidentally exposed to conditions other than those proposed for long-term storage. The excellent thermal stability of Cervarix™ is therefore compatible with worldwide supply of the vaccine.

# THE INFLUENCE OF ELEVATED OXYGEN PARTIAL PRESSURE ON SPECIFIC VIRUS PRODUCTIVITIES IN AN INFLUENZA VACCINE PROCESS

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To increase process yields in virus vaccine production in mammalian cell culture high cell densities are of prior interest. As an example we investigate an influenza vaccine production process [1]. Madin Darby canine kidney cells (MDCK) were cultivated on Cytodex1 microcarriers and later infected with influenza A virus. To achieve maximum productivity in such a process one has to verify that specific virus productivities should remain unchanged with increasing cell densities. In high-density cell cultures process parameters such as medium and oxygen supply also have to be considered. Simpson et al. [2] reported for a hybridoma cell line in mAb production that the amount of apoptotic cells increased due to several stress factors, i.e. under elevated oxygen partial pressure.
Additionally, apoptosis is induced in the host cell during influenza virus infection (Takizawa et al. [3]). The virus induced apoptosis plays a crucial role during influenza virus infection as shown by Ludwig et al. [4]. Therefore, an investigation concerning the correlation between oxygen partial pressure induced apoptosis and virus replication during vaccine manufacturing has been carried out.

Here, we present results obtained for high-density cell culture in small scale bioreactors. Cell numbers of 6.0 to 8.0 x $10^6$ cells/mL were achieved in cultivations with microcarrier concentrations of 10 g/L. Cultivations at 40 % of air saturation and above 150 % of air saturation will be compared with respect to cell numbers on microcarriers, virus titres, level of apoptosis (TUNEL assay), and glycosylation of viral haemagglutinin proteins (capillary gel electrophoresis–laser induced fluorescence (CGE - LIF)). First results indicate that apoptosis induced by elevated oxygen partial pressure can be beneficial for increasing virus yields.

[1] Genzel et al.; 2004. Vaccine 22: 2202-2208
[2] Simpson et al.; 1997. Biotechnology and Bioengineering 54: 1- 16
[3] Takizawa et al.; 1993. Journal of General Virology 74: 2347 - 2355
[4] Ludwig et al.; 2006. Cellular Microbiology 8(3): 375 - 386

**PREDICTIVE MODELING IN ROTAVIRUS-LIKE PARTICLES PRODUCTION: IMPROVING UPSTREAM AND DOWNSTREAM PROCESSING DESIGN**

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Optimisation of rotavirus-like particle (RLP) production in insect cells is extremely complex. Multiplicity of infection (MOI), individual protein production ($VP_2$, $VP_6$ and $VP_7$) and correspondent assembly into a triple layered particle (RLP) are major challenges for the upstream processing. Concomitantly, the downstream processing of RLPs faces several setbacks due to the complexity of the cell cultured bulk imposing a number of purification steps. Thus, strategies to suppress product-derived contaminants (trimers of $VP_6$ and double layered particles – DLP) negatively impacting the overall process were designed based on novel mathematical and analytical tools. Such a rational approach integrating up- and downstream phases will be shown to improve global yields.

To maximise product synthesis, the starting point consisted in evaluating the effect of MOI on final $VP_2$ protein synthesis (used as a model for the remaining VPs). A stochastic/structured mathematical model developed in our group, combining an explicit stochastic infection process with intracellular mass action kinetics, was used. Final productivities were shown to be a compromise between high intracellular $VP_2$ templates, obtained at high MOIs, and high infected cell densities, obtained at low MOIs. In fact, an optimal midterm MOI between 0.01 and 1 pfu.cell$^{-1}$ maximised process productivity. This strategy, further applied to the remaining VPs, will allow the establishment of optimal operational conditions for maximisation of RLPs production yields confirming the relevance of mathematical models in process development. Moreover, these results highlight the relevance of having accurate analyticals for viral stock titers estimation as MOI variations may compromise process optimisation. Several titration methods were compared. The techniques that better combined all important variables (accuracy, cost per titration, titration time and labor intensity) and therefore proved reliable for titer estimation were the $TCID_{50}$, MTT and flow cytometric assays with intra-variabilities and costs per titration of 19% and 13.5€, respectively.

Complementarily to this upstream optimisation, the design of an efficient downstream process route is equally important. A novel strategy using anion-exchange membrane chromatography (Sartobind$^{TM}$ D membrane adsorber from Sartorius Stedim Biotech) was evaluated as these permitted high fluxes, rapid processing, little buffer consumption and a validation-free environment. Screening studies performed to elucidate the effect of the ionic strength and the pH of the equilibration buffer on the RLP capture efficiency allowed for the definition of best operational conditions. Moreover, steric mass action formalism was implemented for the prediction of the adsorption/elution profile of RLPs demonstrating to be an important mathematical tool for process optimisation. At the end, global downstream processing yields were improved by 46%.

Mathematical tools were here demonstrated to play a determinant role in optimising RLPs production. The predictive capacity of the models presented show their utility in both up- and downstream phases reducing labor time and process costs, and improving yields.

# DEVELOPMENT OF A CELL CULTURE PRODUCTION PLATFORM FOR COLD-ADAPTED LIVE ATTENUATED INFLUENZA VACCINE (CAIV) STRAINS OF FLUMIST®: EFFECTS AND INTERACTIONS OF MEDIUM COMPONENTS, TRYPSIN, AND DIFFERENT INFLUENZA VIRUSES IN PROCESS PRODUCTIVITY

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A cell culture based manufacturing process is highly desirable for influenza vaccine production, especially during a pandemic when an additional quantity of vaccine is demanded in a short period of time. Similar to seasonal influenza vaccine strains, pandemic influenza virus strains can also propagate in selected mammalian and avian cells. Seasonal influenza vaccines consist of three different seasonal strains which are selected each season based on the circulating influenza strains. Hence, one of our challenges is to develop a robust cell culture based platform process that will require only minimal process changes for any new seasonal virus strain and for future unknown pandemic influenza viruses.

In this communication, we report our efforts in developing a cell culture based platform process for production of cold-adapted live attenuated influenza virus strains (FluMist®) using Madin Darby Canine Kidney (MDCK) cells. These cells were cultured on microcarriers in proprietary serum-free media and infected with influenza vaccine strains. Using this process, we consistently obtained infectious peak virus titers > 8.3 log10 Focus Forming Units per ml (FFU/ml) for the three 2007/2008 seasonal strains and representative prototype pandemic vaccine strains. However, we also observed that the overall range of peak titers spanned from 7 to 9 log10 FFU/ml for influenza virus vaccine strains tested thus far. In order to reduce the variability in the peak titers obtained for different viruses and to improve the overall virus productivity, we evaluated several process improvement strategies. As reported by other authors, exchanging the cell growth medium (CGM) with fresh and leaner medium during infection greatly improve virus titers. Nevertheless, a systematic evaluation of all CGM components failed to identify the components that were fully inhibitory to virus replication, although some caused a delay in virus production. In addition, isolation of macromolecules secreted by MDCK cells in the 10-100 kDa or >100 KDa range were found not to inhibit virus replication. Trypsin-like activity was confirmed to be critical for influenza A viruses but not for influenza B viruses, but the optimal range of trypsin concentration seems to be dependent on the specific strain.

In conclusion, our results provide critical insights regarding cell culture medium factors that affect influenza vaccine productivity, which can be used to guide medium/process optimization efforts and ultimately increase vaccine productivity.

# DEVELOPMENT OF A CELL CULTURE PRODUCTION PLATFORM FOR COLD-ADAPTED LIVE ATTENUATED INFLUENZA VACCINE (CAIV) STRAINS OF FLUMIST®: ACCELERATED DEVELOPMENT OF A FULLY DISPOSABLE PHASE I CLINICAL MANUFACTURING PROCESS

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Current influenza vaccines are manufactured using embryonated hens' eggs. The potential occurrence of a pandemic outbreak of avian influenza might reduce or even eliminate the existing supply of eggs, leaving the human population at risk. Also, the current egg production technology is intrinsically cumbersome and not easily scalable to provide a rapid worldwide supply of vaccine.

In this work, the development of a platform process for cell culture production of a cold-adapted live attenuated influenza vaccine using a novel disposable Single Use Bioreactor (SUB) is presented. The SUB is designed as a stirred tank reactor for animal cell culture, with PID control of pH, DO, agitation, and temperature, similar to traditional glass or stainless steel bioreactors. The main product contact component of the SUB is a disposable plastic bag, thus, eliminating the need for in-situ cleaning, cleaning validation and sterilization of culture vessels. In addition, it allows quick turn-around for bioreactor use, since a production run can be stopped and reinitiated in just a few hours.

Influenza virus production was carried out at 25 to 30L scale in the SUB by growing Serum Free (SF) Madin-Darby Canine Kidney (MDCK) cells on microcarriers, in proprietary serum-free media and then infecting the cells with FluMist® vaccine strains. MDCK cell growth performance was evaluated in the SUBs and found to be affected primarily by the agitation rate. After optimization of various process parameters, the cell growth rates reached the same level as that in 3L glass bioreactors. Additional process parameter changes, particularly those affecting virus production conditions, resulted in high yield of all three 2006/2007 seasonal FluMist® virus strains: A/New Caledonia/20/99 (H1N1 strain), A/Wisconsin/67/05 (H3N2 strain) and B/Malaysia/2506/04 (B strain) with each strain producing peak infectious virus titers ≥ 8.6 log10FFU/mL in the SUB at 30 L scale. This is equivalent to >1 million doses in a single reactor run (approximately 40 doses per mL of harvest fluid). The accelerated development of the above disposable platform has enabled the start of a GMP campaign in just 5 months from the initiation of the first SUB run performed at development stage, and to successfully produce influenza vaccine clinical trial material.

In conclusion, these results demonstrate the ability of the developed platform process in SUBs to produce influenza virus vaccine at high titer, and also its suitability for rapid implementation in clinical material production settings under cGMP.

**CAPILLARY ELECTROPHORESIS FOR THE DIFFERENTIATION OF DOUBLE-LAYERED AND TRIPLE-LAYERED ROTAVIRUS-LIKE PARTICLES**

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Triple-layered rotavirus-like particle (tlRLP) production requires the simultaneous recombinant expression of three proteins, VP2, VP6 and VP7. These proteins assemble into triple, double or single-layered RLP. Successful production and application of rotavirus-like particles as vaccines requires the differentiation and quantification of each of these structures, as complete tlRLP are needed for an adequate immune response during vaccination. In the past, we developed a method to quantify double-layered (dl) RLP utilizing gel-permeation chromatography. However, this method cannot be utilized to quantify and differentiate dlRLP and tlRLP, as their difference in size is only 5 nm. Here, we report the use of zone capillary electrophoresis to achieve this task. We were able to successfully separate and quantify both dlRLP and tlRLP. Detection limits of 1.6 and 2.6 µg/mL were obtained for dlRLP and tlRLP, respectively. This methodology allows the precise determination of the amounts of each RLP that are used for immunization, permitting the establishment of more rigorous immunization protocols with RLP and evaluation of the outcome.

**Poster Number 29**

**DIFFERENTIAL EXPRESSION AND FUNCTIONAL ANALYSIS OF *E. RUMINANTIUM* PROTEINS: IDENTIFICATION OF POTENTIAL ANTIGENS FOR A SUBUNIT HEARTWATER VACCINE**

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Heartwater, a tick-borne disease of domestic and wild ruminants, is caused by the intracellular rickettsia *Ehrlichia ruminantium* (ER), posing a major constraint to livestock production throughout sub-Saharan Africa and some Caribbean islands.

Despite the recent advances in genomics towards the screening of novel antigens to be used as vaccine candidates against heartwater, it is clear that the genome sequence *per se* does not provide information regarding to the temporal expression and function of the genes that are transcribed. Thus, to investigate the presence of proteins in a given biological compartment, at a specific time and in a defined environment, proteome studies are required. Herein, we have studied the expression of several ER genes (map1, map1-2, map1-6, map1+1, map1-14 and cpg2) along the bacterial developmental cycle and attempted to compare protein expression profiles between ER elementary bodies (infectious, EB) and reticulate bodies (non-infectious, RB). For this, infected BAE cell monolayers were harvested at different times post-infection and total ER protein extracts were prepared. The antigen identification was performed using Western Blot and immunofluorescence techniques methodologies.

We could identify 5 of 6 ER proteins initially proposed, i.e., MAP1, MAP1.6, MAP1.2, CpG2 and MAP 1.14. Interestingly, from 5 to 48 hpi, most of these antigens are not detected. This time window corresponds to the lag and early exponential phase of ER growth and, hypothetically, when the transition from EB to RB would occur. These proteins are detected again at approx 72 hpi (which corresponds to the mid-exponential phase in ER growth development) and it is maintained until 120 hpi, when ER elementary bodies are released from the host cells. Furthermore, some of the expressed proteins were found to be glycosylated (MAP1 and MAP1-2) and MAP1 proteins appears to be organised as a trimeric form, which exists only in EB.

From the results obtained herein, it was also clear that the developmental cycle of ER in BAE cells is an asynchronous process, resulting in a heterogeneous population of bacterial morphologies with different protein expression profiles. Furthermore, we have begun to identify what are likely to be significant proteins involved in the process of redifferentiation of RB into mature, infective EB, such as MAP1.

Overall, this knowledge not only will contribute to a better understanding of heartwater pathology as it can provide additional information on potential immunogens to develop a more effective vaccine against heartwater.

**CHARACTERIZATION OF A CANCER VACCINE BASED ON VERY SMALL SIZE PROTEOLIPOSOME (VSSP) OBTAINED BY DIFFERENT FORMULATION PROCESSES.**

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The VSSP is a nano-particle obtained by incorporation of GM3 ganglioside into the outer membrane complex of Neisseria meningitides. This nano-formulation has showed an effective adjuvant capacity in the dendritic cell activation and generation of CTL response to peptide and protein antigen. In this study, we evaluated the physicochemical properties (ganglioside/protein ratio, protein profile, size distribution, and zeta potential) and biological activity the VSSP nano-particle obtained by two different formulation processes using Dialysis and Ultrafiltration. The dialyzed VSSP showed a ganglioside/protein ratio of 1.04 with trimodal particle size distribution while VSSP obtained by ultrafiltration showed a ganglioside/protein ratio of 0.69 with monomodal particle size distribution. However, the size of the main peak was 24 nm in both conditions. The protein profile and antitumoral activity were also similar.

# THE SILVER ANNIVERSARY OF CLINICAL PROTEIN PRODUCTION FROM RECOMBINANT CHO CELL CULTURE

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Chinese Hamster Ovary (CHO) cells have been successfully employed to manufacture not only glycosylated antibodies, growth factors, and enzymes, but also glycosylated protein subunit vaccines.

In 1983, Genentech initiated the first human clinical trial of a therapeutic protein made in recombinant CHO cell culture. At the time, many viewed recombinant cell culture as a production method of last resort, certainly not one suitable to make a high-dose therapeutic. With low peak cell densities and low specific productivities in medium containing expensive animal-derived components, final product concentrations at harvest were low, typically below 1 mg/L. Furthermore, there were regulatory risks, as the safety of products from continuous aneuploid cell lines (like CHO) had yet to be established.

Twenty five years later, recombinant CHO cell culture has emerged as one of the dominant methods for production of recombinant glycosylated proteins. The story behind this emergence should be viewed as one of the great case studies in new technology development.

In this presentation, data from 25 years of clinical production is analyzed to derive the CHO cell culture equivalent of 'Moore's Law' for semiconductors. Cell culture engineers are pitted against semiconductor and agricultural engineers. Progress rates for firms new to the field are distinguished from the rates for experienced firms. For batch and fed-batch CHO cell cultures used for clinical production, titers at harvest for experienced firms have doubled every 2 -3 years. Progress rates are compared against the historical benchmarks for penicillin and used to estimate a future maximum titer of 20 g/L.

Participants will be recruited for a new, parallel study regarding progress rates in the manufacture of viral vaccines.

**A CELL-CULTURE-BASED PLATFORM FOR VIRAL VACCINE PRODUCTION FOR HUMANS**

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Cell-culture-based processes are a suitable alternative for viral vaccine production since they provide several advantages over other manufacturing methods. Since Vero cells are currently considered an acceptable cell substrate to express a variety of viruses, we developed a virus production platform using Vero cells adapted to grow in suspension in a serum free medium.

After adapting anchorage dependent Vero cells to grow as a free-cell suspension, the cell line was capable of proliferating in a chemically defined synthetic medium. The production yields of vesicular stomatitis virus, herpes simplex virus 1 and polio virus 1 were evaluated using batch cultures of adherent Vero cells growing in T-flasks and the cells growing in suspension in spinner flasks. The effects of the multiplicity of infection (m.o.i), harvesting time, cell concentration and use of serum were evaluated. Under optimized conditions, similar production yields were achieved with both systems.

Perfusion cultures in a bioreactor were performed in serum free media. When cell density reached 1-2x$10^7$ cells/ml cultures were infected with vesicular stomatitis virus and polio virus 1. Infection with vesicular stomatitis virus at m.o.i = 0.02 resulted in a viral titer of 2x$10^{10}$ $TCID_{50}$/ml at 25 h post infection. The viral specific production was 4 times higher than the obtained in batch experiments. In addition, infection with polio virus 1 at m.o.i. = 0.5 produced a significant 30 fold increment in viral titer.

These results constitute valuable information for the development of a low-cost large-scale process using a suspension culture of Vero cells for producing viral vaccines.

**Poster Number 33**

# RAPID MYCOPLASMA TESTING: THE HYMY™ ASSAY COMBINES AMPLIFICATION OF VIABLE MYCOPLASMAS IN BROTH CULTURE WITH SIGNAL DETECTION BY QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

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Safety testing of biopharmaceutical products is vitally important prior to administration of product to patients. However, speed from manufacturing of the drug to having it released and available for the clinic is also very important and anything that can be done to reduce this time is of benefit to both the Manufacturer and the patient. Mycoplasma testing is an important part of the safety testing for biopharmaceuticals and currently takes 28 days. This timescale is not conducive for obtaining the rapid lot release testing results needed for biopharmaceutical products having short shelf-lives or for which there is a high market demand. This article seeks to explain the advantages of a more rapid test that has been developed to meet the need for speed to market, but which provides the same confidence in the results and therefore the safety of the therapeutic. The decrease in time for a critical biosafety test brings huge advantages in manufacturing when rapid and accurate testing results are required for lot release testing, raw material testing and in-process intermediate testing.

# COMPARISON OF DENGUE-2 VIRUS PRODUCTION IN VERO CELLS UNDER SERUM-FREE AND SERUM-CONTAINING CONDITIONS

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The dengue virus belongs to the *Flaviviridae* family and occurs in 4 different serotypes (dengue-1 to dengue-4). Dengue fever is an emerging disease that has experienced a significant worldwide increase in incidence in the last years, representing a public health concern in many tropical and subtropical countries. Among the diseases transmitted by mosquitoes, dengue is currently the most common worlwide, with approximately 100 million cases every year and a mortality rate of 2.5%. In spite of its impact on public health, no effective vaccine against dengue fever is yet available on the market.

Vero cells are accepted for the production of human vaccines and are presently used in the commercial production of different vaccines, such as polio and rabies. However, one of the main regulatory concerns in this field is related to the use of animal serum and animal-derived components as culture medium additives. Therefore, in this work we compared the production of dengue-2 virus (DEN-2) in a serum-containing medium (DMEM + 5% fetal bovine serum) and in a commercial serum-free medium (VP-SFM, Gibco). A factorial statistical experimental design methodology was used to evaluate the influence of TOI (1-3 days) and MOI (0.002-0.02) on virus titer. Experiments were carried out both in T-flasks and in spinner flasks using Cytodex-1 microcarriers, and virus titer was monitored for 7 days post-infection.

The results showed that the influence of MOI and TOI on virus titer varies according to the culture medium used. In most experiments, DEN-2 virus titer presented an increasing trend in the whole evaluated period. However, for large MOI values (0.02), a maximum virus production was observed 5 days post-infection in the serum-free medium regardless of the time of infection, and in the serum-containing medium this peak at 5 dpi occurred only when a large MOI value was combined with a large TOI (3 days). For the serum-supplemented medium, the maximum virus titer obtained increased with both MOI and TOI values, whereas for the serum-free medium, virus titer increased with TOI but remained approximately constant with MOI in the range evaluated in this work. In general, the use of the serum-supplemented medium gave higher virus production under all conditions tested. The maximum DEN-2 titer was 4.93E+6 pfu/mL (6.69 in log scale) for the serum-supplemented DMEM medium, and 1.28E+6 pfu/mL (6.11 in log scale) for the serum-free VP-SFM medium.

**Poster Number 35**

# PERFUSION PROCESS FOR HUMAN AND ANIMAL VIRAL VACCINE PRODUCTION IN A SINGLE USE STIRRED TANK BIOREACTOR

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Anchorage-dependent cell lines, such as MDCK, MDBK, Vero, CEF, MRC5, HEK293, etc., are used in biotechnology industry to produce viral vaccines for prophylaxis and viral vectors for gene therapy. At industrial scale, these cells are either cultivated in static mode on multiplate systems (Cell Factories from Nunc, Cell Cube from Corning, etc.) or on microcarriers (porous or non-porous) in suspension in stainless steel stirred-tank bioreactors. Multiplate systems are bulky and require lot of handling operations, whereas stainless steel bioreactors need cleaning and sterilization operations, expensive control validations and high capital investment. For all of the above reasons, the biopharmaceutical companies rely more and more on space and money-saving disposable solutions.

Today, disposable bioreactors are available at different scales, but usually, they are not well adapted to cultivation of animal cell immobilized on microcarriers when a perfusion process is needed because the traditional spin-filter approach cannot be employed.

Herewith we are presenting the case study of cultivation of MDBK (producing BHV viruses) and Vero cells (producing Influenza viruses) cultivated on Cytodex-1 beads (at 6 g/L) inside a Nucleo™ bioreactor. We are comparing the results in cell growth and virus production between single-use and classical stirred tank reactors.

The Nucleo culture bag reactor contains a non-invasive paddle mixing technology, a sparger fixed on the impeller and holds standard DO and pH probes. The perfusion was implemented using an Alternating Tangential Flow system (ATF, Refine Technologies) connected to the reactor, a system based on an external tangential flow screen filter and a diaphragm pump.

After 7 days of cultivation, MDBK cell density has reached up to ca. 150 cells/bead in serum-containing medium (5.8x106 cells/ml) and up to ca. 100 cells/bead for Vero in serum-free conditions. Cells have been infected at confluence, and have respectively produced 1.25x108 Bovine Herpes Viruses/ml for MDBK cells and 3.5x107 Influenza Viruses for Vero cells (while control in STR reactor was 1.15x107 Influenza virus/ml).

These cultivations have established the ability to cultivate immobilized cells in perfusion in a single-use bioreactor for the production of viral vaccines. The combination of these two technologies is attractive for vaccine manufacturing but will reach its full potential when disposable options will be available for the perfusion device as well as for the on-line probes.

# EXPLOITING LYMPHATIC TRANSPORT AND COMPLEMENT ACTIVATION IN TH1 STIMULATING NANOPARTICLE VACCINES

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We are exploring the interface between bionanotechnology and vaccine technology. We are developing a bionanotechnology platform that can deliver antigen and a danger signal to dendritic cells (DCs) that are resident within the lymph node. We engineered antigen-bearing nanoparticle vaccines with two novel features: lymph node-targeting and in situ complement activation. Following intradermal injection, interstitial flow transported our ultra-small nanoparticles (25 nm) highly efficiently into lymphatic capillaries and their draining lymph nodes, targeting half of the DCs there, whereas nanoparticles even 100 nm large were only 10% as efficient. Furthermore, we exploited a polyhydroxylated nanoparticle surface chemistry in order to potently activate the complement cascade via the alternative pathway. Complement-activating nanoparticles that reached lymph node spontaneously generated a danger signal in situ and potently activated DCs. With the model antigen ovalbumin conjugated to the nanoparticles, we demonstrated humoral immunity by measuring IgG antibody titers. The nanoparticle induced potent antibody levels similar to that of potent adjuvants such as aluminum salts and bacterial lipopolysaccharide (LPS). Furthermore, we demonstrated that antigen-bearing nanoparticles could also induce cellular immunity in a complement and lymph node targeting dependent manner. We characterized cellular immunity through measuring a Th1 biased response both on the DC and T cell level. Interleukin 12p40 (IL-12p40) is an essential cytokine secreted by DCs during Th1 activation. Following incubation with polyhydroxylated-nanoparticles pre-treated with serum, DCs produced high levels IL-12p40, approaching that of the potent Th1 activator LPS. Additionally, we show that complement-activating OVA-nanoparticles induce DCs to produce high expression of MHC I. Finally we demonstrate T cell memory of nanoparticle immunized mice through production of inflammatory cytokine interferon (IFN-γ) following re-exposure to an antigenic stimulus. One of the most difficult challenges in vaccine adjuvant technology is overcoming immunotoxicity, often due to overproduction of inflammatory cytokines. Therefore we tested the effect of complement-activating nanoparticles on DCs to produce the pro-inflammatory cytokines TNF-α and IL-6. We observed that there were undetectable levels of both TNF-α and IL-6, indicating that while complement can activate DCs to mature and produce IL-12p40 but they do not stimulate strong pro-inflammatory cytokine expression such as the TLR activators LPS and CpG (unmethylated bacterial DNA).

# MDCK-BASED INFLUENZA PRODUCTION USING CYTODEX 3 IN A WAVE BIOREACTOR

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During the recent couple of years the disposable bioreactor technology has taken the biotech community with storm. However, disposable bioreactors are still not commonly used for microcarrier cultivation, despite the fact that several of the most frequently used cell lines for industrial vaccine production are adherent, e.g. MDCK and Vero, and thus require microcarriers for large scale production.

Here we demonstrate the use of Cytodex™ 3 microcarriers in a process for MDCK-based influenza virus production in a WAVE™ disposable bioreactor system. Attachment of cells to the microcarriers was performed in the WAVE CELLBAG™ using slow rocking during 3-4 hours, after which the rocking was gradually increased concomitant with the cell growth. Oxygen and pH were measured and controlled with a WAVEPOD™ integrated controller. Key metabolites were measured throughout the process. When the cells were near confluence (approx. $10^6$ cells/mL), the culture was infected with Influenza A H1N1/PR8/1934. After incubation at 33 °C for 72 hours, the culture was harvested and the influenza virus and hemagglutinin content analysed with a TCID50 assay, immunoblot and ELISA.

In summary, the WAVE BIOREACTOR™ system was shown to be a versatile system for microcarrier cultivation of MDCK cells and a fast and convenient alternative for influenza production as compared to conventional stirred-tank reactors.

**PROTECTION INDUCED BY PNEUMOCOCCAL SURFACE PROTEIN A (PSPA) IS ENHANCED BY CONJUGATION TO A *STREPTOCOCCUS PNEUMONIAE* CAPSULAR POLYSACCHARIDE**

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The currently available anti-pneumococcal vaccines are based on capsular polysaccharide (PS), plain or conjugated to a carrier protein. Conjugated vaccines are expensive products, especially in the case of pneumococcus, in which reasonable coverage requires from 7 to 13 serotypes. To obtain increased coverage with fewer components, we evaluated the immunogenicity of the Pneumococcal surface protein A (PspA), conjugated to capsular polysaccharide serotype 23F, aiming at induction of an immune response against both components. Mice immunized with PS23F-rPspA1 conjugate produced antibodies against both PS and rPspA1, comparable or slightly higher than that obtained by free PS. The immunized animals challenged with a lethal dose of a virulent strain bearing a homologous PspA, showed that the PS23F-rPspA1 conjugate induced higher survival than rPspA1 alone or in combination with PS. This increased protection was shown to correlate with the enhanced capacity of the antibodies to bind to the pneumococcal surface and to induce complement deposition. Our results indicate that the use of PS-PspA conjugates may be a way to increase coverage against pneumococci with fewer components.

**BIODISTRIBUTION AND TOXICOLOGICAL SAFETY EVALUATION OF ADENOVIRUS TYPE 5 VECTORED VACCINES AGAINST EBOLA AND MARBURG VIRUSES**

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Aims: To recommend safe dosing regimen for delivery of adenoviral vectored vaccines to humans. To identify potential target organs for toxicity to guide clinical safety monitoring plan. To identify potential toxicology parameters that would identify special populations who might be at risk of harm from product.

Methods: Biodistribution of single i.m. injection of $0.89x10^{11}$ VP in rabbits of Adenovirus type 5 (Ad5) vectored Ebola vaccine was determined. 5 animals/gender were sacrificed on Study Day (SD) 9, SD61, and SD93. Tissues were analyzed by quantitative PCR. Toxicological safety evaluations were performed on rabbits receiving repeated i.m. dosing of 1 or $2x10^{11}$ VP Ad5 vectored Ebola vaccines alone or Ad5 vectored Marburg vaccine given as boost to 4 mg DNA plasmid priming series. 5 rabbits/gender sacrificed 2 days and 2 weeks post last injection. Parameters monitored were mortality, morbidity, body weight, food consumption, clinical pathology, body temperatures, ophthalmology, Draize reactogenicity scores, organ weight, and gross and histopathology.

Results: The adenovectors remained primarily at sites of injection in muscle and subcutis and only trafficked to liver and spleen, but no other distal organs, including remaining absent in gonads. Over time, the number of animals with, and copy numbers present in positive tissues declined, demonstrating clearance of the adenovectors. Also, body temperatures were elevated and food consumption was diminished in the day(s) immediately after Ad5 inoculations. Some clinical pathology parameters were impacted. Reversible findings in Draize and histopathology at injection sites seen.

Discussion: Biodistribution and toxicology studies demonstrate that the Ad5 vaccine candidates tested were safe and suitable for investigational clinical use. One of the Ad5 Ebola vaccines has entered clinical trials.

# DEVELOPMENT OF A UNIVERSAL INFLUENZA VACCINE

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Influenza virus is one of the most important respiratory pathogens. In the United States alone, influenza infection (flu) is responsible for 20,000 to 40,000 deaths and over 100,000 hospitalizations annually. Infants, the elderly, and individuals with compromised cardiac, pulmonary, or immune systems are at great risk of serious complications from this virus. Immunization proves to be the most effective measure in preventing infection; vaccines matched antigenically with the circulating viral strains have demonstrated up to 70-90% of efficacy in terms of protecting healthy adults from influenza illness [MMWR (2003);52 (No. *RR-8*)]. The conventional influenza vaccines are composed of inactivated viruses, administered as inactivated whole virions, split or purified viral sub-unit proteins. Recently, cold adapted live influenza vaccines have been licensed in the U.S. for use in populations 5 to 49 years of age. One of the common features shared by all current influenza vaccines is that the vaccines are targeted primarily to the induction of neutralizing antibodies directed against the major viral envelope protein, hemagglutinin (HA). Because HA can readily undergo mutation (antigenic drift), the vaccine formulations need to be evaluated on a yearly basis to match the current circulating strains, and accordingly, immunizations must be performed annually. This requirement imposes a significant burden on vaccine production and supply as well as on vaccination practice.

A vaccine that can provide cross protection against different influenza variants or strains and that does not require frequent updates is highly desirable. Moreover, the immediate availability of such a vaccine in the setting of an influenza pandemic caused by a major antigenic shift would be an extremely valuable public health tool. We'll present a novel strategy in the design of a "universal" conjugate influenza vaccine. Early clinical data suggest the candidate vaccine is both safe and immunogenic.

**PURIFICATION OF RETROVIRUS VECTOR PARTICLES AND IDENTIFICATION OF HOST-ASSOCIATED PROTEINS BY PROTEOMIC ANALYSIS**

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As gene therapy progresses into clinical trials, full characterization of the viral vectors becomes an important issue. Retroviruses are known to acquire minute amounts of host cellular proteins that can be biologically active both on the surface and inside the virion. However, in spite of the widespread use of retroviral vectors both in experimental and clinical studies, the repertoire of host proteins incorporated into MoMLV vector particles remains unexplored. We have developed a rate zonal ultracentrifugation strategy that rendered highly purified retroviral vector preparations suitable for the study of the composition of retroviral vector particles. The high level of purity achieved by this method was demonstrated using a variety of techniques including electrophoretic analyses, size exclusion chromatography, subtilisin digestion and electron microscopy. Proteomic analysis of the purified virions was performed. Viral proteins were fractionated by 1D-SDS-PAGE, in-gel tryptic digested and subjected to liquid chromatography/tandem mass spectrometry analysis (LC-MS/MS). These studies led to the identification of 27 host proteins associated to retroviral particles derived from 293 HEK cells, including 5 proteins previously described as part of wild type MoMLV. Nineteen host proteins identified corresponded to intracellular proteins. A total of 8 host membrane proteins were identified including cell adhesion proteins integrin Î²1 (fibronectin receptor subunit beta) and MFG-E8, tetraspanins CD81 and CD9 and late endosomal markers CD63 and Lamp-2. Immunogold electron microscopy studies confirmed the presence of several host membrane proteins exposed at the vector surface. Membrane proteins are particularly attractive since they can serve as anchoring sites for the insertion of tags for targeting or purification purposes. This work represents an important step forward in the elucidation of retroviral vector composition.

# SPR TECHNOLOGY AS A POWERFUL TOOL TO ACCURATELY DETERMINE INFLUENZA VIRUS CONCENTRATION

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Determining influenza virus hemagglutinin (HA) concentration is critical for the final formulation of the vaccine and for manufacturing process development. The HA concentration is commonly determined by single radial immuno-diffusion assay (SRID) as recommended by the European Pharmacopoeia and WHO. However, the SRID assay has a number of disadvantages as it is laborious and has a low detection range inducing high variation. Thus, SRID is not an optimal method for neither batch release analysis of the vaccine nor in process development. Surface Plasmon Resonance (SPR) technology monitors the interactions of a molecule on the surface of a chip, and holds the potential of being a highly sensitive, less time consuming and more accurate methodology.

In this study an analytical method using Biacore™ was developed with the goal of receiving a robust and accurate assay for three influenza virus types (A/H1N1, A/H3N2 and B) with lower detection limit than SRID (<5µg HA/ml). The robustness, accuracy, precision and detection range of the three influenza virus Biacore assays were investigated and compared to SRID analysis. It was found that the Biacore assay has high specificity, showing the potential of a future vaccine batch release assay.

Furthermore, the assay was shown to have higher precision for in process samples and a higher detection range with a limit of detection ~1 log lower than SRID. Therefore, the Biacore assay is among the most compelling analytical tools in process development for influenza vaccines.

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