Strategies for improved Adenovirus and PPR vaccine production in different cell lines:

from bioprocess development to final formulation

Ana Carina Santos Ferreira da Silva

Dissertation presented to obtain the Ph.D degree in Engineering and Technology Sciences, Biotechnology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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By Ana Carina Silva

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Composite image illustrating different steps of viral production processes

By Ana Carina Silva

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Foreword

The present thesis dissertation is the result of several years of research at the Animal Cell Technology Unit of ITQB-UNL/iBET, Oeiras, Portugal, under the supervision of Dr Paula M. Alves and Dr Pedro E. Cruz. It gave me the opportunity to work in the challenging fields of cell culture and viral production for vaccination and gene therapy applications.

This thesis intends to identify and investigate some of the major bottlenecks in the upstream bioprocesses for virus production. Two products were selected based on their importance in the gene therapy field (Adenovirus) and the challenging demand of developing a thermostable vaccine for the eradication of Peste des Petits Ruminant Virus in Africa. These products characteristics and complexity of their production systems were the starting point for the different challenges faced during this work. The main focus of this dissertation was based on the selection of the best cell host for production and the best formulation to maintain virus product quality during storage.

À Filipa

Aos meus Pais

À memória dos meus Avós Álvaro e Maria

“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning.”

Albert Einstein

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E por fim, aos meus avós, aos meus pais e à minha irmã, a quem dedico esta tese. À memória dos meus avós Maria e Álvaro por tudo o que me ensinaram e por todo o apoio que jamais irei esquecer. Aos meus pais por me apoiarem sempre. Pelo carinho, pelo optimismo, pelo encorajamento. À Filipa a minha manita, por me ouvir, por me apoiar, por estar ao meu lado sempre…

Abstract

Viral products, including viral vaccines and viral vectors, are important tools for disease prevention and therapy. Viral vaccines, such as attenuated or inactivated rabies virus, influenza virus or hepatitis virus vaccines are powerful tools to limit the number of serious viral infections and pandemics. This type of biopharmaceuticals are used to improve human quality of life with vaccination being the most cost-effective method available for preventing economic losses and increasing the lifespan of both people and livestock. On the other hand, adenoviruses, adeno-associated viruses and retroviruses are among the viral vectors currently being developed for delivering genetic material to the target cell in a growing number of gene therapy clinical applications.

The production of viral products is typically based on cell culture, the exception being the use of eggs as a mean of virus replication prior to inactivation as in the case of influenza virus vaccine. Several factors must be considered when selecting an appropriate cell line for a given bioprocess. Productivity, is of course, important but other aspects such as process simplicity and cost, scalability, reproducibility, safety, regulatory history, production platform compatibility, and availability of preferred production media may also play a decisive role. As a result, new and improved platforms are continuously being developed propelled by the need to increase and improve product availability and quality.

Two different viral systems, Adenovirus vectors (AdV) and Peste des Petits Ruminants (PPR) virus, were selected for this thesis due to their contrasting position on the viral products domain. AdV belong to the most widely used tools for the delivery of transgenes into cells *in vitro* and *in vivo* with applications ranging from gene therapy and cancer therapy, to the development of prophylactic and therapeutic vaccines. Adenovirus vectors have been extensively used for research both at preclinical and clinical levels (the majority in phase I/II clinical trials) and two products based on adenovirus for the treatment of cancer have been approved in China. Consequently, the market need for adenovirus is increasing, creating a demand for new production methodologies of high quality, high efficacy vectors.

Contrary to AdV, which are non-enveloped viruses, PPR is an enveloped virus which makes it very sensitive to temperature. PPR has become the next

veterinary disease on target for elimination, after the successful eradication of Rinderpest in 2011. Several efforts are being taken to develop DIVA vaccines (which enable differentiation between infected and vaccinated animals) for PPR and to improve the efficacy of the current attenuated vaccine, via the increase of its thermal stability.

Both products share similar challenges but there are also product specific bioprocess bottlenecks that need to be addressed. In this thesis, several cell lines and culture strategies were evaluated in their capacity to produce AdV and PPR vaccine. Several cell culture and virus production parameters were assessed namely multiplicity of infection (MOI), cell concentration at infection (CCI), the use of serum-free media and the scalability of the production process for both systems. Being the thermal stability a very important factor for vaccine use, approaches to improve the current PPR vaccine are also presented together with their application in one of the production laboratories.

In **Chapter 1**, the state of the art on animal cell culture for virus production is presented. The several bioengineering challenges affecting upstream processing are reviewed. A special focus is also made to the factors affecting virus stability during production and storage.

In **Chapter 2**, a newly developed human amniocyte-derived cell line (1G3 cells) was evaluated in its ability to produce AdV and in comparison to the human cell line HEK293 typically used for those viral vectors. The impact of MOI, CCI and harvesting time (TOH) on AdV production were evaluated in shake flask and bioreactor. Overall, the AdV infection experiments with 1G3 cells showed that (i) infection was most efficient at a MOI of 5 infectious particles/cell; ii) infection done at a CCI $3x10^6$ cell/mL in bioreactor provided the higher virus titers; iii) the preferable time of harvest was 48 hours post-infection, corresponding to the higher virus concentration, mainly in the intracellular fraction. This strategy allowed a 3-fold increase in the AdV volumetric productivity obtained in batch culture in comparison to HEK293 cells. The human amniocyte-derived cell line (1G3 cells) showed to be a valid alternative substrate for adenovirus vectors production.

The aim of **Chapter 3** was to study the impact of adenovirus infection in two producer cell lines (HEK293 and 1G3 cells) metabolism, and gain further insights into the cell density effect which occurs at higher densities for 1G3 cells. The exometabolome of both cell lines, infected at different concentrations in shake flask and bioreactor cultures, was analyzed by ^{1}H-NMR spectroscopy. The cultures supernatant was analyzed along time to evaluate how the virus manipulation of the cell metabolism evolves in both cell lines.

In **Chapter 4**, two scalable processes were evaluated as possible alternatives for the currently used production process of PPR attenuated vaccine that uses monolayers of Vero cells grown in roller bottles or static flasks, i.e. processes with very limited scalability. i) Microcarrier cell culture technology to produce PPR vaccine in Vero cells and ii) single cell suspension cultures of BHK-21A or HEK293 cells, significantly simplifying the existing production process were studied.

Two strategies to improve the stability of the current PPR vaccine are presented in **Chapter 5**. Firstly, new formulations based on the Tris buffer were tested, with and without the addition of sucrose and trehalose and compared with the formulation normally used to stabilize the vaccine, the Weybridge medium. The results show a virus half-life of 21 hours at 37ºC and 1 month at 4ºC for the Tris/trehalose liquid formulation. In the lyophilized form, the formulation was able to maintain the viral titer above the $1x10^{4}$ $TCID_{50}$/mL (>10 doses/mL) for at least 21 months at 4ºC (0.6 log reduction), 144 hours at 37ºC (0.6 log reduction) and 120 hours at 45ºC (1 log reduction). Secondly, a strategy based on culture medium composition manipulation aiming at improving the intrinsic PPR vaccine stability was also evaluated. The addition of 25 mM fructose resulted in a higher virus production (1 log increase) with higher stability (2.6 fold increase compared to glucose 25 mM) at 37ºC.

Chapter 6 presents the technology transfer operation of a new candidate formulation to the production platform of PPR vaccine preformed at National Veterinary Institute (NVI) in Ethiopia. The results showed an increased thermal stability of the vaccine, especially at 37 and 45ºC, as expected from results obtained in **Chapter 5** which validates the data obtained.

Chapter 7 discusses the implications of the findings and main achievements of this thesis. The challenges and hurdles associated with viral manufacture using animal cell cultures and their effects during the production process are discussed.

Overall, this thesis describes novel and/or alternative production processes for two of complex biopharmaceuticals (Adenovirus vectors and PPR attenuated vaccine) taking into account the current bottlenecks in their production. It contributes to improve current knowledge in vaccine process development trying to move towards more robust, cost-effective and safe (RCA formation for AdV) manufacturing. Furthermore, the results obtained for these two biopharmaceuticals can provide useful hits for production of other similar products or even be used as a starting point for development of new production platforms.

Finally, it is important to highlight that the results obtained in **Chapter 5** were used to transfer the developed formulation to the production of the PPR vaccine in Ethiopia (**Chapter 6**), with the results showing an improvement in the stability of the vaccine produced thus demonstrating a successful technology transfer operation.

Resumo

Os vectores virais e vacinas virais têm vindo a ganhar uma importância clínica cada vez mais relevante. Os vectores virais como o adenovírus, os vírus adeno-associados ou os retrovírus, são ferramentas que têm vindo a ser desenvolvidas para entregar material genético à célula alvo em terapia génica. As vacinas virais, como as vacinas da raiva (atenuada ou inativada), da gripe ou da hepatite, são ferramentas poderosas para limitar o número de infeções virais graves e pandemias. Estes tipos de produtos biofarmacêuticos são utilizados para melhorar a qualidade de vida dos seres humanos sendo a vacinação o método disponível mais rentável para a prevenção de perdas económicas e aumento do tempo de vida do homem, mas também dos animais.

Muitos fatores têm que ser considerados na escolha da linha celular apropriada para qualquer bioprocesso. A produtividade é, naturalmente, uma consideração importante, mas outras questões incluem a facilidade e economia da sua utilização, a escalabilidade, a reprodutibilidade, a segurança, historial regulamentar, a compatibilidade com a plataforma de produção e a disponibilidade dos meios de produção pretendidos. Assim, são continuamente desenvolvidas novas e melhores plataformas para expandir e melhorar a disponibilidade e qualidade do produto.

Dois sistemas virais, vectores adenovirais (AdV) e a vacina da *Peste des Petits Ruminants* (PPR), foram selecionados para esta tese devido à sua posição contrastante no domínio dos produtos virais. Os AdV pertencem às ferramentas mais usadas na entrega de transgenes em células *in vitro* e *in vivo* com aplicações desde a terapia génica e a terapia contra o cancro, até ao desenvolvimento de vacinas profiláticas e terapêuticas. Este vectores têm sido extensivamente usados na investigação tanto nos níveis pré-clínico e clínico (na sua maioria em fase I/II) e dois produtos baseados em adenovírus para o tratamento de cancro foram recentemente aprovados na China. Por isso, as necessidades de mercado para os vectores adenovirais estão a aumentar, criando a necessidade de metodologias de produção de vectores concentrados com a garantia de qualidade e eficácia.

Ao contrário dos AdV, que são vírus sem envelope e com ciclo celular lítico, o virus PPR têm envelope que os torna mais sensíveis à temperatura. A PPR tornou-se a próxima doença veterinária alvo para a completa eliminação, após a

erradicação da *Rinderpest*, conseguida em 2011. Vários esforços têm sido efetuados para o desenvolvimento de vacinas marcadoras de doença (capazes de diferenciar entre animais vacinados de animais infetados) e para melhorar a eficácia da vacina atenuada atual, passando pelo aumento da sua estabilidade térmica.

Nesta tese, são apresentadas linhas celulares alternativas e estratégias de cultura para a produção de dois produtos virais relevantes, os vectores adenovirais e o vírus da PPR atenuado. Vários parâmetros de cultura celular e produção viral são alvo de estudo nomeadamente a multiplicidade de infeção (MOI), a concentração na altura da infeção (CCI), a utilização de meios sem soro e a escalabilidade do processo para ambos os sistemas. Sendo a estabilidade térmica um factor muito importante na utilização de vacinas, alternativas para melhorar a atual vacina da PPR são também descritas juntamente com a sua aplicação num dos laboratórios de produção.

No **Capítulo 1**, faz-se uma revisão do estado da arte em cultura de células de animais para a produção de vírus. Os vários desafios de bioengenharia que afetam o processo de produção são apresentadas. Um foco especial é feito também sobre os factores que afetam a estabilidade dos vírus durante a produção e o armazenamento.

No **Capítulo 2**, é avaliada a aplicabilidade de uma linha celular humana derivada de amniócitos recentemente desenvolvida (células 1G3), em comparação com a linha celular normalmente utilizada, na produção de AdV, as células humanas HEK293. Os efeitos da MOI, da CCI e o tempo de recolha (TOH) na produção de AdV foram analisados em frascos agitados e em biorreator. Em geral, as experiências de infeção com as células 1G3 mostraram que (i) a infeção por AdV foi mais eficiente a uma MOI de 5 partículas infecciosas/célula, ii) a infecção feita a uma CCI 3 em biorreator, originou os títulos virais mais elevados, iii) o tempo de recolha preferível foi de 48 horas pós infecção, correspondente à concentração mais elevada de vírus, principalmente na fração intracelular. Esta estratégia permitiu um aumento de 3 vezes na produtividade volumétrica de AdV obtidos na cultura em *batch*, em comparação com células HEK293. A linha celular derivada de amniócitos humanos (células 1G3) mostrou ser uma alternativa válida para a produção de vectores adenovirais.

O principal objectivo do **Capítulo 3** foi estudar o impacto na infeção por adenovírus no metabolismo de duas linhas de células de produção (as células HEK293 e as 1G3), e tentar obter conhecimentos sobre o efeito da concentração celular que ocorre a valores superiores nas células 1G3. O metaboloma extracelular exometabolome de ambas as linhas celulares, infetadas a diferentes concentrações em frasco agitados e em biorreatores, foi analisado por espetroscopia de ^{1}H-RMN. Os sobrenadantes das culturas foram analisadas ao longo do tempo para avaliar o modo como a manipulação pelo vírus faz evoluir o metabolismo em ambas as linhas celulares.

No **Capítulo 4**, dois processos escalonáveis foram avaliados como possíveis substitutos para a produção da vacina atualmente utilizada contra a *Peste des Petits Ruminants* (PPR) usando monocamadas de células Vero cultivadas em frascos estáticos ou frascos de rolantes. Na primeira aplicou-se a tecnologia de cultura de células em microsuportes para a produção da vacina PPR usando células Vero e na segunda foram usadas como alternativa culturas células em suspensão, as células BHK-21A ou HEK293, o que pode simplificar significativamente o processo de produção atualmente existente.

Duas estratégias para melhorar a estabilidade da vacina atual PPR são apresentadas no **Capítulo 5**. Em primeiro lugar, novas formulações baseadas no tampão Tris foram testadas com e sem a adição de sacarose e trealose e comparadas com a formulação normalmente utilizada para estabilizar a vacina, o meio *Weybridge*. Os resultados mostram um tempo de semi-vida do vírus de 21 horas a 37 ºC e 1 mês a 4 ºC para a formulação de Tris/trealose líquida. Sob a forma liofilizada, a formulação foi capaz de manter o título viral acima de 1×10^4 $TCID_{50}$/ml (> 10 doses/mL) durante pelo menos 21 meses a 4 º C (0,6 log perdidos), 144 horas a 37 ºC (0,6 log perdidos) e 120 horas a 45 ºC (1 log perdidos). Em segundo lugar, foi também avaliada uma estratégia baseada na manipulação da composição do meio de cultura com o objetivo de melhorar a estabilidade intrínseca da vacina PPR. A adição de 25 mM de frutose resultou na produção de vírus mais elevada (aumento de 1 log) e com uma estabilidade superior (2,6 vezes de aumento, em comparação com glucose 25 mM) a 37 ºC.

O **Capítulo 6** apresenta os resultados da operação de transferência de uma nova formulação candidata na plataforma de produção da vacina contra a PPR realizados no Instituto Nacional de Veterinária (NVI), da Etiópia. Os resultados mostraram um aumento da estabilidade térmica da vacina, especialmente a 37 a 45 ºC, tal como o esperado a partir dos resultados obtidos no **Capítulo 5** validando assim os dados obtidos.

O **Capítulo 7** discute os resultados apresentados nos outros capítulos e resume as principais implicações identificando as principais metas atingidas nesta tese. A identificação dos novos desafios e obstáculos associados ao fabrico viral utilizando culturas de células animais são discutidos.

Em resumo, esta tese descreve novos processos de produção alternativos para dois biofármacos complexos tendo em consideração os desafios atuais de produção e propondo novas estratégias para superá-los. Contribui-se para melhorar o conhecimento atual do desenvolvimento de processos permitindo avançar para métodos de produção mais robustos, eficazes, económicos e seguros (sem formação de virus recombinantes no caso dos Adenovirus). Os resultados obtidos para os dois produtos podem ser úteis para a produção de outros produtos semelhantes, ou até um ponto de partida para o desenvolvimento de novas plataformas de produção.

Finalmente, é de notar que os resultados descobertos no **Capítulo 5** foram utilizados para transferir a formulação desenvolvida para a produção da vacina PPR na Etiópia (**Capítulo 6**) onde os resultados obtidos mostraram uma melhoria da estabilidade da vacina produzida demonstrando assim o sucesso desta operação de transferência de tecnologia.

Thesis publications

Original articles

1. Silva, A.C., Teixeira, A. and Alves, P.M, “Impact of Adenovirus infection in host cells metabolism evaluated by ^{1}H-NMR spectroscopy“ (*submitted*).

2. Silva, A.C.*, Simão, D*, Küppers, C.*, Lukas, T. *, Sousa, M.F.Q., Cruz, P.E, Kochanek, S., Carrondo, M.J.T. and Alves, P.M., “., “Human amniocyte-derived cells are a promising cell host for adenoviral vector production under serum free conditions” *Biotechnology Journal 2015,* 10(5): 760-71 (*These authors contributed equally).

3. Silva, A.C**.,** Yami, M., Libeau, G., Carrondo, M.J.T. and Alves, P.M, “Testing a new formulation for Peste des Petits Ruminants vaccine in Ethiopia”, *Vaccine* 2014, 32 (24), 2878–2881.

4. Silva, A.C., Carrondo, M.J.T. and Alves, P.M., “Strategies for improved stability of Peste des Petits Ruminants Vaccine”, *Vaccine* 2011, 29, 4983– 4991.

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- Silva, A.C.*, Peixoto, C. *, Lukas, T. *, Küppers, C. *, Cruz, P.E., Alves, P.M and Kochanek, S., “Adenovirus vector production and purification”, *Current Gene Therapy* 2010, 10 (6), 437-455. (*These authors contributed equally)

Book Chapters

- Silva, A.C**.,** Roldão, A., Teixeira, A., Fernandes, P., Sousa, M.F.Q., Alves, P.M. 2015. “Cell immobilization for the production of viral vaccines” In: Al-Rubeai, Mohamed (Ed.), Animal Cell Culture, Cell Engineering, Vol. 9, Springer.
- Silva, A.C**.,** Fernandes, P., Sousa, M.F.Q., and Alves, P.M., “Scalable production of adenovirus vectors” (2014) In: Adenovirus – Methods and Protocols, Chillón, Miguel; Bosch, Assumpció (Eds.), Series: Methods in Molecular Biology, Volume 1089, 3rd Edition, Springer.
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- Fernandes P., Silva A.C., Coroadinha A.S., Alves P.M., "Upstream Bioprocess for Adenovirus Vectors, In Adenoviral Vectors for Gene Therapy, Elsevier 2016 (*in press*).
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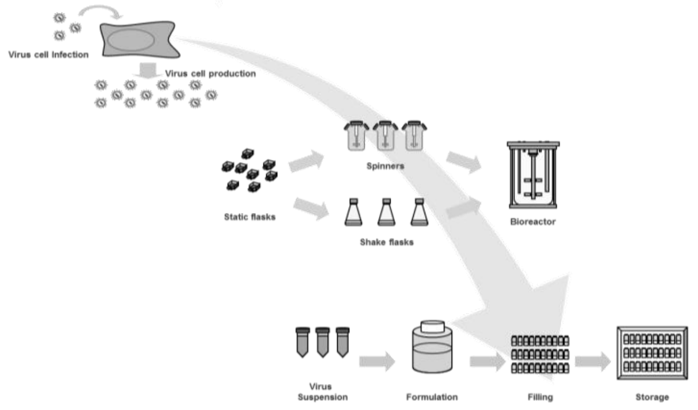
Abbreviations

Abbreviation	Full form
AdV	Adenovirus
ACF	Animal Component Free
BHK	Baby Hamster Kidney
BUGS	Buffered gelatine-sorbitol
CCI	Cell Concentration at Infection
CHO	Chinese Hamster Ovary
CPE	CytoPatic Effect
DMEM	Dulbecco's Modified Eagle's Medium
DO	Dissolved Oxygen
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
Glc	Glucose
Gln	Glutamine
GMEM	Glasgow Minimum Essential Medium
HEK	Human Embryonic Kidney
hpi	hours post infection
IP	Infectious Particles
Lac	Lactate
LM	Lactalbumin hydrolysate-Manitol
LS	Lactalbumin hydrolysate-Sucrose
MEM	Minimum Essential Medium
MOI	Multiplicity Of Infection
PBS	Phosphate-Buffered Saline
PPR	Peste des Petits Ruminants
PPRV	Peste des Petits Ruminants Virus
RCA	Replication Competent Adenovirus
RP	Rinderpest
SLAM	Signalling lymphocytic activation molecule
Suc	Sucrose
TCID50	Tissue Culture infectious Dose at 50%
TD	Trehalose Dihydrate
TOH	Time Of Harvest
Tre	Trehalose
VP	Virus Particles
WBM	Weybridge medium

Chapter 1

Introduction



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Adenovirus vector production and purification, Silva, A.C., Peixoto, C., Lukas, T., Küppers, C., Cruz, P.E., Alves, P.M and Kochanek, S., (2010) *Current Gene Therapy,* 10 (6), 437-455.

Viruses and Virus-Like Particles in Biotechnology: Fundamentals and Applications, Roldão, A., **Silva, A.C.,** Mellado, M.C.M., Alves P.M., and Carrondo, M.J.T. (2011) The Biophysical Basis | In: Murray Moo-Young (ed.), Comprehensive Biotechnology, Second Edition, volume 1, pp. 625–649. Elsevier

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1. Introduction

Manufacturing of biopharmaceuticals involves the use of cell culture systems as a unique method of product development. These types of products are difficult to obtain by conventional pharmaceutical development processes. Cell culture is used in a wide range of applications such as development of model systems for research, analysis of cellular structures and their mechanisms, drug discovery and evaluation, stem cell research and production, genetic engineering and production of biologics such as antibodies, vaccines, viral vectors for gene therapy and proteins. The increase demand of animal cell bioprocess for biological products created great scientific and economical interest in research and development for this area. Each cell line shows its specificities, and its use for viral production has advantages but also disadvantages compared to the other cell lines. Based on a thorough understanding of the pros and cons of individual host cells for process performance, properties of other cell lines, specific aspects of virus-host cell interaction and process options can be better evaluated. Overall, a systematic comparison of cell lines might help to develop process optimization strategies, as well as to assess quality differences concerning the virus strains and antigens produced.

1.1. Milestones of Cell Culture and viral applications

The need for cell culture, especially at large scales, became apparent with the need for viral vaccines. The major epidemics of polio in the 1940s and 1950s promoted a lot of effort to develop an effective vaccine. When it was finally demonstrated in 1949 that poliovirus could be grown in cultures of human cells, considerable interest was shown to develop large quantities of the polio vaccine using cell culture. The polio vaccine, produced from inactivated virus, became one of the first commercial products of cultured animal cells. After that it was realized that passage in cell culture was also a mean of attenuation, presumably by fortuitous selection of mutants better adapted to replication *in vitro* than in living hosts. Cell culture also permitted conscious selection of mutants by isolation of single clones and by incubation at temperatures below the normal temperature of the host. Thus, the period

between 1950 and 1980 saw the development of numerous attenuated viral vaccines, including those for polio (Sabin oral), measles, rubella, mumps and varicella.

In 1961 Howard Temin discovered that specific genetic mutations could be inherited as a result of virus infection [1]. Based on his experimental observations he concluded that chicken cells infected with the Rous sarcoma virus (RSV) stably inherited viral specific gene mutations that contained the information for the generation of RSV progenies. It became apparent that viruses possessed properties that could be very useful in delivering genes into cells of interest and that genetic engineering may become a new approach for treating genetic diseases. In 1966, Edward Tatum published a paper evoking the effectiveness of viruses to be used in somatic-cell genetics and possibly in genetic therapy [2].

The first officially approved clinical protocol to introduce a foreign gene into humans was approved by the Recombinant DNA Advisory Committee (RAC) in December 1988. In that, no actual therapy was proposed, but instead, S.A. Rosenberg aimed at using gene marking techniques to track the movements of tumour-infiltrating blood cells in cancer patients [3].

Currently, more than 1800 approved gene therapy clinical trials worldwide have been conducted or are still ongoing. Up to date, cancer is by far the most common disease treated by gene therapy. It composes over 60% of all ongoing clinical gene therapy trials worldwide [4, 5]. Three gene therapy products for cancer have recently received market approved, two in China and one in Europe, increasing the confidence in further research and commercialization.
Vaccine development has led to eradication of smallpox and the elimination of poliomyelitis and measles from large parts of the world, saving millions of lives. The use of vaccines has also contributed to promote animal health by preventing disease outbreaks that can have a devastating effect on animal production and contributed to the eradication of Rinderpest in 2011. Meanwhile several other types of vaccines have been obtained and are in commercial manufacture or are under development, being most of them viral based. In **Table 1.1** are highlighted some of the milestones during the history of cell culture, vaccine development and gene therapy.

Table 1.1: Some important events in the historical of cell culture and vaccine development and gene therapy products.

Year	Vaccine Event
1796	Edward Jenner had first speculated that protection from smallpox disease could be obtained through inoculation with a related virus, vaccinia or cowpox. He tested his theory by inoculating eight-year-old James Phipps with cowpox pustule liquid recovered from the hand of a milkmaid, Sarah Nelmes.
1879	Louis Pasteur created the first live attenuated bacterial vaccine (chicken cholera).
1884	The first live attenuated viral vaccine (rabies) was developed by Louis Pasteur, using desiccated brain tissue inactivated with formaldehyde.
1885	Louis Pasteur first used rabies vaccine in humans.
1896	Cholera and typhoid vaccines were first developed.
1897	Plague vaccine was introduced, following the preparation of anti-plague horse serum at the Pasteur Institute by Alexandre Yersin.
1918	Whole-cell inactivated bacterial vaccine (pertussis whooping cough).
1923	Toxoid vaccine prepared from inactivated bacterial toxins (diphtheria).
1930	Cell culture was developed and shown to be able to grow virus, thus paving the way for the subsequent production of viral vaccines.
1931	Freeze-dried vaccine approved by FDA (smallpox).
1949	Viral vaccine produced in vitro with non-neural human cells by Enders (polio).
1951	The first human cell line, HeLa was established.
1954	Freeze drying process for smallpox vaccine greatly improved by Collier to allow global distribution.
1955	The first polio vaccine was licensed - an inactivated poliovirus vaccine (IPV) produced in vitro with primary monkey kidney cells pioneered by Dr. Jonas Salk.
1962	Vero cells were originally isolated from the kidney of a normal (*i.e.*, non-diseased) adult African green monkey by Y. Yasumura and Y. Kawakita at the Chiba University in Chiba, Japan.
1962	Attenuated Measles Vaccine Developed (Rubeovax) was developed by Maurice Hilleman and colleagues.
1962	Human diploid cell line (WI-38) established by Hayflick.
1977	Last case of smallpox outside of the laboratory.
1980	Rabies human diploid-cell vaccine (Imovax Rabies by Mérieux and Wyvac by Wyeth) were licensed.
1980	The World Health Assembly certified the world free of naturally-occurring smallpox.
1981	The first hepatitis B viral vaccines (plasma-based), developed by Merck and also by the Pasteur Institute, were licensed.
1986	Recombinant virus-like particle hepatitis B vaccine (Recombivax HB by Merck) was licensed.
1987	Protein-conjugated *Haemophilus influenzae* type b vaccine (PRP-D, ProHibit by Connaught) was licensed.
1988	First officially approved clinical protocol to introduce a foreign gene into humans was approved.
1998	The first vaccine for the prevention of HIV/AIDS (Aidsvax) entered Phase III trial, the first large-scale human trial of an HIV vaccine.
2003	First gene therapy product approved in China, Gendicine™.
2007	FDA approves first U.S. vaccine for humans against the avian influenza virus H5N1.
2009	FDA approved new vaccine (Cervarix, GlaxoSmithKline) for the prevention of cervical cancer.
2011	The World Organisation for Animal Health declares the Eradication of Rinderpest, the second disease in history to be fully wiped out.
2012	FDA approved first seasonal influenza vaccine manufactured using cell culture technology (Flucelvax, Novartis).
2012	First gene therapy product approved in Europe, Glybera.

(Source: http://www.immunize.org/timeline/ ; http://www.historyofvaccines.org/content/timelines/all)

2. Viral products

Viruses are amongst the simplest biological systems. They behave as intracellular parasites and contain a limited set of genes that encode information for their replication, encapsidation and cell-to-cell propagation. Viruses have been under considerable investigation as a model to study the basic concepts of biology and virology and due to their impact in animal and human health. The main areas of application of viruses include vaccine development and gene therapy [6].

2.1. Types of viruses

The nature of the viral genome has many implications on the life cycle of the virus. Viral genome consists of one or more fragments of single-stranded (ss) or double-stranded (ds) RNA or DNA. Most DNA and positive (+) ssRNA viruses rely on host cell machinery for initiating the replication and transcription of viral genome, not requiring de novo synthesis of other virus gene products. Others, such as dsRNA or negative (-) ssRNA viruses have to deliver into the cell their own polymerase for the synthesis of viral proteins essential for initiating viral replication as host cells do not possess the machinery to cope with dsRNA or negative (-) ssRNA. In addition, many dsRNA viruses deliver their genome within a protein capsid to avoid contact between the dsRNA and the cell cytosol [6].

2.1.1. Virus with DNA

A DNA virus has DNA as its genetic material and replicates using a DNA-dependent DNA polymerase. The nucleic acid is usually dsDNA; ssDNA is less common as during replication ssDNA typically expands to dsDNA. This class of viruses belong to group I or group II of the Baltimore classification system for viruses [7]. Group I virus includes the *Adenoviridae*, *Herpesviridae* (herpes simplex virus - HSV) and *Baculoviridae* (*Autographa californica* multicapsid nucleopolyhedrovirus) families while group II virus includes the *Parvoviridae* and *Circoviridae* families (**Table 1.2**). Although group VII viruses such as hepatitis B (hepatitis B virus - HBV) contain a DNA genome, they are not considered DNA viruses according to the Baltimore classification, but rather

reverse transcribing viruses because they replicate through an RNA intermediate [6].

Table 1.2: List of virus with DNA genomes.

Virus Family	Virus Examples (common names)	Virion (naked/enveloped)	Capsid symmetry	Nucleic acid type	Group
Adenoviridae	Adenovirus, Canine adenovírus type 2, Human adenovirus type 2 and 5	Naked	Icosahedral	ds	I
Baculoviridae	*Autographa californica* multicapsid nucleopolyhedrovirus	Enveloped	Helical	ds	I
Circoviridae	Transfusion transmitted virus	Naked	Icosahedral	ss circular	II
Hepadnaviridae	Hepatitis B virus	Enveloped	Icosahedral	ds-RT	VII
Herpesviridae	Herpes simplex virus, Varicella-zoster virus, Cytomegalovirus, Epstein-Barr virus, Varicella virus	Enveloped	Icosahedral	ds	I
Papillomaviridae	Human papillomavirus, Bovine papillomavirus	Naked	Icosahedral	ds circular	I
Parvoviridae	Parvovirus B19, Porcine parvovirus, Adeno-associated virus	Naked	Icosahedral	ss	II
Polyomaviridae	Polyomavirus; JC polyomavirus, Simian virus 40, Goose hemorrhagic polyomavirus, Murine polyomavirus	Naked	Icosahedral	ds circular	I
Poxviridae	Smallpox virus, Vaccinia virus	Complex coats	Complex	ds	I

ds: double stranded; ss: single stranded; RT: reverse transcriptase. (Source: [6])

2.1.2. Virus with RNA

Conversely, an RNA virus has RNA as its genetic material. This nucleic acid is usually ssRNA but may be dsRNA. The International Committee on Taxonomy of Viruses classifies RNA viruses as those that belong to group III, group IV or group V of the Baltimore classification system for viruses [8] and does not consider viruses with DNA intermediates as RNA viruses. These viruses are included in group VI and possess ssRNA genomes that replicate using reverse transcriptase. Viruses such as the human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and rous sarcoma (RS) virus (members of the *Retroviridae* family) are included in this group [6].

2.1.2.1. Group III: dsRNA viruses

DsRNA viruses represent a large group of pathogens whose genome can be monopartite or segmented up to 12 fragments. These viruses do not release the free dsRNA genome in infected cells and require that transcription and synthesis of new dsRNA genomes take place in confined environments. Reovirus and Rotavirus, members of the *Reoviridae* family are included in this group (**Table 1.3**).

2.1.2.2. Group IV: (+) ss RNA viruses

Contrasting with the dsRNA or the (−) ssRNA viruses, the genomes of (+) ssRNA viruses are infectious. Icosahedral (+) ssRNA viruses represent a large fraction of all viruses known and include important human pathogens; poliovirus, human rhinoviruses, hepatitis A virus, Norwalk group of viruses, astroviruses, and members of the alpha- and flaviviruses which carry an RNA-containing nucleocapsid are some examples. Unlike (−) ssRNA viruses, the nucleoproteins responsible for protecting the genome from unspecific cellular RNA binding are not expressed in (+) ssRNA viruses. Thus, the synthesis of progeny viruses requires that the capsid proteins of these viruses specifically package the viral RNA genome while excluding the ubiquitous cellular RNA. Group IV includes the *Flaviviridae* (hepatitis C virus - HCV), *Coronaviridae* (severe acute respiratory syndrome virus – SARS virus), *Picornaviridae* and *Caliciviridae* families (**Table 1.3**).

Table 1.3: List of virus with RNA genomes.

Virus Family	Virus Examples (common names)	Virion	Capsid symmetry	Nucleic acid type	Group
Arenaviridae	Lymphocytic choriomeningitis virus	Enveloped	Complex	(-) ss	V
Astroviridae	Astrovirus	Naked	Icosahedral	(+) ss	IV
Birnaviridae	Infectious bursal disease virus	Naked	Icosahedral	ds	III
Bornaviridae	Borna disease virus	Enveloped	Helical	(-) ss	V
Bunyaviridae	Hantaan virus, Rift valley fever virus	Enveloped	Helical	(-) ss	V
Caliciviridae	Norwalk virus, Hepatitis E virus, Norovirus	Naked	Icosahedral	(+) ss	IV
Coronaviridae	Severe acute respiratory syndrome virus	Enveloped	Helical	(+) ss	IV
Filoviridae	Ebola virus, Marburg virus	Enveloped	Helical	(-) ss	V
Flaviviridae	Dengue virus, Hepatitis C virus, Yellow fever virus	Enveloped	Icosahedral	(+) ss	IV
Orthomyxoviridae	Influenza A, B and C virus,	Enveloped	Helical	(-) ss	V
Paramyxoviridae	Measles virus, Newcastle disease virus, Rinderpest virus, Peste des Petits Ruminant	Enveloped	Helical	(-) ss	V
Picornaviridae	Rhinovirus, Hepatovirus, Poliovirus, Hepatitis A virus	Naked	Icosahedral	(+) ss	IV
Reoviridae	Reovirus, Rotavirus, Bluetongue virus	Naked	Icosahedral	ds	III
Retroviridae	HIV , SIV, Lentivirus, Moloney murine leukemia virus	Enveloped	-	ss-RT	VI
Rhabdoviridae	Rabies virus	Enveloped	Helical	(-) ss	V
Tetraviridae	*Nudaurelia capensis* virus	Naked	Icosahedral	(+) ss	IV
Togaviridae	Rubella virus, Alphavirus	Enveloped	Icosahedral	(+) ss	IV

ds: double-stranded; (-) ss: negative single-stranded; (+) ss: positive single-stranded; RT: reverse transcriptase. (Source: [6])

2.1.2.3. Group V: (-) ss RNA viruses

Negative ssRNA viruses are classified into seven families: *Rhabdo*-, *Paramyxo*-, *Filo*-, *Borna*-, *Arena*-, *Bunya*- (Hantaan virus and rift valley fever virus - RVFV) and *Orthomyxoviridae* (Influenza). The first four families are characterized by non-segmented genomes. The remaining three have genomes comprising two, three and six to eight (-) sense RNA segments, respectively. The large group of (-) sense RNA viruses includes: i) highly prevalent human pathogens such as respiratory syncytial virus (RSV), parainfluenza and Influenza viruses; ii) two of the most deadly human pathogens, Ebola and Marburg viruses; iii) viruses with a major economic impact on the poultry and cattle industries, namely the Newcastle disease virus (NDV), rinderpest virus and Peste des Petits Ruminant virus (**Table 1.3**) [6].

2.2. Gene therapy

Gene therapy involves the use of nucleic acids (DNA or RNA) for the treatment, cure or prevention of human disorders. Depending on the type of disease, this can be achieved either by delivery of a functional, therapeutic gene as a substitute for the defective or missing endogenous counterpart or by reducing the levels of a harmful defective gene product, using various sophisticated tools including naked oligonucleotides, viral and non-viral vectors.

In the late 1970s and early 80s, the emergence of techniques for subcloning mammalian genes into prokaryotic plasmids and bacteriophages were correctly foreseen as precursors of techniques for human gene therapy. Parallel investigations on the biology of avian and murine onco-retroviruses led to the development of retroviral vectors, which began to be used in the mid-80s as a tool for gene transfer into mammalian cells. The first human trial of gene transfer was carried out in the late 80s for the treatment of patients with advanced metastatic cancer. The process consisted in introducing the gene coding for resistance to neomycin into human tumor-infiltrating lymphocytes by retroviral-mediated gene transduction, before their infusion into patients; thus, using the new gene as a marker for the infused cells [3]. Since then, there has been a remarkable expansion in the number of vector systems available to

express human genes directly associated with disease states for therapeutic purposes (**Table 1.4**).

Table 1.4: Vectors used for Gene therapy

Virus Family/Vector	Advantages	Limitations
Adenoviridae/ **Adenovirus**	- High efficiency - Transduces quiescent and dividing cells - >30 kb transgene capacity - Easy to produce in high titers	- Coxsackie adenovirus receptor-dependent transduction - Immunogenic - Existing humoral response to certain serotypes
Retroviridae/ **Murine leukaemia virus**	- Broad tropism - Low immunogenicity - Stable integration	- Insertional mutagenesis - Unable to transduce quiescent cells - Inactivation by serum
Retroviridae/ **Lentivirus**	- Low immunogenicity - Stable integration to quiescent cells	- Insertional mutagenesis - Potential risk of recombination of pathogenic vector (HIV)
Parvoviridae/ **Adeno-associated virus**	- Transduces quiescent and dividing cells - Very long expression time - Non-pathogenic, low immunogenicity - Broad tropism	- Very small transgene capacity - Insertional mutagenesis may be problem
Alphaviridae/ **Semliki forest**	- High titer - Broad host range - Efficient transgene expression	- Low transgene capacity - Highly cytotoxic - Short term expression
Alphaherpesviridae/ **Herpes simplex-1**	- Broad host range, - High titer, - Large capacity	- Latent wild type-viral activation risk, - Antigenic
Baculoviridae/ **Autographa californica multicapsid nucleopolyhedrovirus**	- High titer - Large transgene capacity - Easy production - Non-pathogenic	- Limited transduction - Production in insect cells - Unstable genome
Non-viral vectors	- Easy preparation, - non-pathogenic	- Limited transduction - Bacterial contaminants

Gene therapy trials using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID) constitute one of the most successful applications of gene therapy to date. X-SCID is a disease in which the patient has neither cell - mediated immune response nor is able to generate antibodies. A high rate of immune system reconstitution was observed in patients treated in the X-SCID gene therapy trials [9]; but, 5 out of more than 20 patients developed a leukemia-like illness, of which 4 fully recovered after conventional anti-leukemia treatment [10, 11].

Despite these results, gene therapy trials to treat X-SCID due to deficiency of the adenosine deaminase enzyme continue with relative success in the USA, France, UK, Italy and Japan. In the last decade, the process of retroviral vector production has been under considerable investigation as it presents many difficulties, mainly due to vector instability and low cell productivities hampering the attainment of high viral titers. Strategies based on the manipulation of sugar carbon sources [12, 13], lipids [14], temperature [15] or osmotic pressure [13] used in bioreaction and on the establishment of pioneering packaging cell lines such as 293 FLEX [16] and Flp293A [17] show potential to increase the yields of infectious retroviral vectors. This will allow the generation of high quality clinical preparations for gene therapy applications.

Adenovirus (AdV) vectors are also efficient vehicles for delivering nucleic acids into mammalian cells (see more information of this vectors in section 3.2 below). The human adenovirus type 2 and 5 are the most used vector backbones for adenovirus-mediated gene transfer. However, due to a number of significant disadvantages such as the need to immunosuppress or tolerize patients to a potentially debilitating virus [18, 19], vectors from different serotypes or derived from non-human adenovirus (bovine, sheep and birds) were developed [20, 21]. Recently, canine adenovirus serotype 2 (CAV-2), produced in dog kidney cells, have been gaining increasing attention due to their emerging potential for the study of the pathophysiology and potential treatment of neurodegenerative diseases like Parkinson's, Alzheimer and Huntington, amongst others. The first clinical trial using recombinant adenovirus was carried out in 1993 with cystic fibrosis (CF) patients [22]. Two years later, the first recombinant adeno-associated virus trial was initiated in CF patients [23]; trials in hemophilia B patients commenced shortly after [24]. Inevitably, lentivirus and recombinant herpesvirus vectors have also entered in clinical trials [25-27]. All four viral vector systems mentioned above are highly efficient systems for gene transfer and expression *in vivo* in non-dividing cells. In fact, more than 50% of all viral vectors currently undergoing clinical trials are adenovirus (22.2%), retrovirus (18.4%), adeno-associated virus (6%), Lentivirus (5%) or herpes simplex virus (3.2%) (**Figure 1.1**).

Due to their inability for replication and absence of toxicity in mammalian

cells, baculoviral vectors have emerged as gene therapy vehicles for the treatment of a wide range of human diseases. Recently, a genetically modified recombinant baculovirus encoding for a cherry-red fluorescent protein under the control of a strong mammalian cell promoter (cytomegalovirus promoter) proved to be effective in transducing a human liver carcinoma cell line, HepG2 [28].

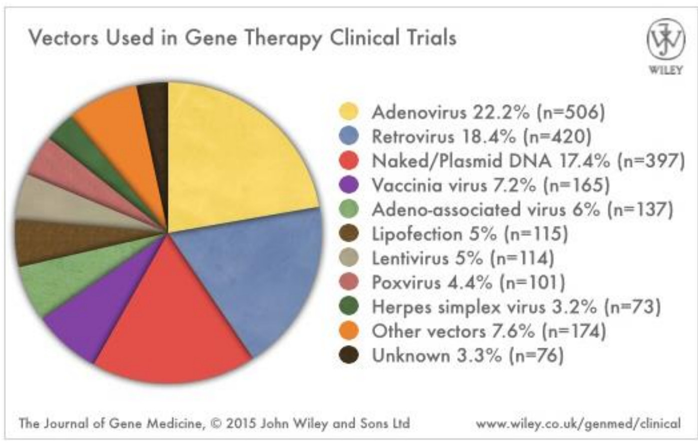

Figure 1.1: Vectors used in Gene Therapy Clinical Trials.

(Source: Journal of Gene Medicine Clinical Trial Database http://www.abedia.com/wiley/index.html)

Other studies indicate that baculoviruses show promising gene expression efficiencies in liver [29], skeletal muscle [30], brain [31] and eye [32]. Importantly, baculoviral vectors present similar efficiencies to adenoviral vectors in transducing human smooth muscle cells, human cardiomyocytes and fibroblasts [33]. The major challenge is the production of high recombinant baculovirus titers [34]. Although metabolic engineering approaches have shown to improve baculovirus titers at high cell densities [34, 35], platforms for the production of baculoviral vectors to be used in gene therapy clinical trials have not yet been implemented.

The first gene therapy products entered the market through China: Gendicine™ (SiBiono GeneTech) in 2003 and Oncorine™ (Shangai Sunway Biotech) in 2006 for cancer treatment. Gendicine™ is a replication defective AdV5 vector expressing p53 from a Rous sarcoma virus (RSV) promoter, and is approved for use in head and neck squamous cell. Oncorine™ is an oncolytic virus product derived from AdV5 vector for nasopharyengeal carcinoma combined with chemotherapy. Noteworthy in this case is the fact that the

China's State Food and Drug Administration (SFDA) approved Gendicine without data from a standard phase III clinical trial [36]. Consequently, soon after the approval of Gendicine™, there was discussion about the efficacy of the treatment [37]. In 2004, Ark Therapeutics Group plc received the first commercial GMP Certification in the EU for the manufacture of commercial supplies of gene-based medicines (Cerepro®). Cerepro® is an adenoviral vector harboring the gene for the Herpes simplex virus thymidine kinase (HSV-tk), developed by Ark Therapeutics Group plc and intended for the treatment of malignant brain tumors. In 2008, Cerepro® became the first and so far the only adenoviral vector that has completed a phase III clinical trial [38].

Finally in 2012, Glybera® (uniQure biopharma, Netherlands) received market approval for the treatment of lipoprotein lipase deficiency (LPLD) using an adeno-associated vector, being the first gene therapy product approved in the Western World. Hopefully, these achievements will facilitate further research and commercialization of valuable gene therapy products.

2.3. Vaccine development

The development process for vaccines is unique. Vaccine development is highly capital intensive and risky. Given the importance of safety with biologics, the vaccine industry is highly regulated. Vaccine development is normally carried out in an iterative fashion. Less than one in ten vaccine candidates actually achieves the market. The high attrition rate is due to the unpredictability of the biological microorganisms needed to produce vaccines, and to the uncertainty of how immune system will process and react to the vaccine antigen. Some vaccine candidates may produce appropriate levels of immune response, but induce important adverse reactions. Other vaccine candidates may be safe, but ineffective at preventing diseases. With the current tendency to combine several antigens into a single vaccine, the challenges associated with developing safe and effective vaccines are even greater. Research to discover new vaccine antigens and novel approaches to immunization usually takes several years, and costs tens of millions of dollars. Once a discovery is made, several developments must be undertaken to reach the licensing stage.

Vaccines based on live viruses have been traditionally effective and relatively easy to produce. The elimination of smallpox was accomplished through mass vaccination with the live vaccinia virus, a mildly pathogenic animal virus related to smallpox. Likewise, live attenuated vaccines are well tolerated and highly immunogenic. The live attenuated poliovirus vaccine developed by Dr. Albert Sabin in 1961 eradicated poliovirus disease in the Western hemisphere and drastically reduced their incidence rate worldwide. Vaccines against infectious diseases such as the yellow fever, typhoid fever, mumps and shigella are also based on lived attenuated viruses. The attenuation of viruses is accomplished through one of the following methods: 1) attenuation of the pathogen by physical means; 2) selection of naturally occurring mutants that lead to infection with abortive replication of the pathogen while retaining its immunogenicity. Inactivated (killed) vaccines can also stimulate a protective immune response. The inactivated poliovirus vaccines (IPOL, Sanofi Pasteur SA), Influenza vaccines (Fluarix, GlaxoSmithKline) and typhoid fever vaccines (Typhim Vi, Sanofi Pasteur MSD) constitute some examples. The disease causing organism is inactivated with chemicals such as formaldehyde; the main drawback of these vaccines is that they require boosting for continuous, efficient immune response. Nowadays, using molecular biology and DNA manipulation methods, it is possible to express protective proteins in adequate live vectors with the purpose of designing live vaccines against various types of pathogens. In addition, the development of reverse genetics systems for the recovery of viruses from cDNA has made it possible to rapidly generate recombinant attenuated derivatives.

Advances in molecular biology allowed also the development of platforms for the production of virus-like particles (VLPs) as vaccines against emergent diseases. Virus-like particles are composed by viral structural proteins that, when expressed in recombinant systems, form multi-protein structures mimicking the organization and conformation of authentic native viruses but lacking the viral genome. **Table 1.5** lists some examples of current licensed vaccines and presents the different types of vaccines and corresponding advantages and disadvantages.

Table 1.5: A summary of vaccine classifications.

Vaccine Type		Licensed Vaccines	Advantages	Disadvantages
Live attenuated vaccines	virus	Smallpox, polio, measles, mumps, rubella, chicken pox, rotavirus, shingles, influenza, yellow fever	- Produce a strong immune response - Often give lifelong immunity with one or two doses	- Remote possibility of reversion to wild type - Must be refrigerated to stay potent
	bacterium	Tuberculosis, typhoid		
Inactivated vaccines	virus	Inactivated polio, Japanese encephalitis, hepatitis A, Influenza (seasonal and pandemic), rabies	- Safer and more stable than live vaccines - Do not require refrigeration: more easily stored and transported	- Produce a weaker immune response than live vaccines - Usually require additional doses, or booster shots
	bacterium	Whole cell pertussis		
Toxoid vaccines	Purified protein toxoid	Tetanus, anthrax and diphtheria	- Teaches the immune system to fight off the bacterial toxins	- Necessary an adjuvant to increase immunogenicit y - Several doses
Subunit vaccines	Purified virus-like particles (VLPs)	Hepatitis B and human papillomavirus	- Target to very specific parts of the microbe - Fewer antigens, so lower chance of adverse reactions	- When developing a new vaccine, identifying the best antigens can be difficult and time consuming
	Purified protein	Acellular pertussis		
	Purified polysaccharide	Pneumococcal for adults, typhoid		
Conjugated vaccines	Polysaccharide conjugated to carrier protein	Pneumococcal for infants, haemophilus type B, bacterial meningitis	- Allow infant immune system to recognize certain bacteria	- Can only be used for bacteria (?)
DNA vaccines		In development /clinical testing	- Produce a strong antibody and cellular immune response - Relatively easy and inexpensive to produce	- Still in experimental stages
Recombinant vector vaccines		In development /clinical testing	- Closely mimic a natural infection, stimulating a strong immune response	- Still in experimental stages

(Adapted from: [39]; [40]; [41])

2.3.1. Veterinary versus Human Vaccines

The burden of infectious diseases in livestock and other animals continues to be a major constraint to sustained agricultural development, food security, and participation of developing and in-transition countries in the economic benefits of international trade in livestock commodities [42].

The criteria for successful animal or veterinary vaccines can be very different from those for human vaccines depending on the animal groups under consideration. For example, criteria for companion animal vaccines are similar to those for human vaccines in that the health and welfare of the individual animal are primary concerns. The main objective of livestock vaccines, on the other hand, is to improve overall production for the primary producers, and the cost-benefit resulting from vaccination is the bottom line for this industry [43].

The process of developing veterinary vaccines has both advantages and disadvantages over human vaccine development. On the one hand, the potential returns for animal vaccine producers are much less than those for human vaccines, with lower sales prices and smaller market sizes, resulting in a much lower investment in research and development in the animal vaccine area than in the human vaccine area, although the complexity and range of hosts and pathogens are greater. For example, the market size for the recently launched human vaccine (Gardasil) against papillomavirus and cervical cancer is estimated to be greater than 1 billion U.S. dollars, while the most successful animal health vaccines (e.g., against foot-and-mouth disease [FMD] virus in cattle and Mycoplasma hyopneumoniae in pigs) enjoy a combined market size that is 10 to 20% of this figure.

On the other hand, veterinary vaccine development generally has less stringent regulatory and preclinical trial requirements, which can make up the largest cost in human vaccine development, and a shorter time to market launch and return on investment in research and development. In contrast to human vaccine development, veterinary scientists are also able to immediately perform research in the relevant target species. This is an obvious advantage over human vaccine development, as experimental infections, dose-response studies, and challenge inoculations need not be carried out in less relevant models [43].

3. Adenovirus

Adenoviruses were first discovered half a century ago by Rowe and colleagues while they were trying to culture adenoid tissue in the laboratory [44]. Ever since, these viruses have been the object of intense study, mainly as a model system for basic eukaryotic cellular processes such as transcription, RNA processing, DNA replication, and translation.

3.1. Adenovirus Biology

Adenovirus is a non-enveloped, icosahedral virus of 60–90 nm in diameter with a linear, double-stranded DNA genome of 30–40 kb. The capsid is composed of 240 hexon capsomeres forming the 20 triangular faces of the icosahedron, and 12 penton capsomeres with spike-shaped protrusions located at the 12 vertices (**Figure 1.2**).

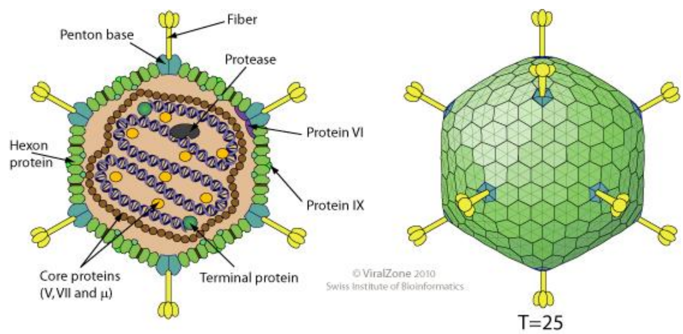

Figure 1.2: Adenovirus particle structure.
(Source: ViralZone:www.expasy.org/viralzone, Swiss Institute of Bioinformatics)

In addition to the major capsid protein (hexon) and the penton base and fiber proteins associated to form the penton capsomere, several hexon- and penton-base-associated proteins are stabilizing components of the capsid. Histon-like viral core proteins are associated with the DNA packaged within the capsid. Altogether, the adenoviral particle has a molecular weight of about 150 MDa. The terminal globular domain or ''knob'' region of the homotrimeric protruding fibers of the AdV capsid is responsible for the primary virus attachment to the cellular receptor, the coxsackie- and adenovirus receptor (CAR). All serotypes except group B adenoviruses have been shown to use CAR as a primary docking receptor. Following the initial attachment, the interaction

between an RGD-motif exposed in a protruding loop of the penton base protein with a cell surface integrin molecule (αvβ1, αvβ3, or αvβ5) serving as secondary or internalization receptor triggers the virus uptake by clathrin-dependent receptor-mediated endocytosis. The endosomal uptake of the virus and release into the cytoplasm is accompanied by a stepwise dismantling of the capsid, leading to the microtubuliassisted transport and finally very efficient delivery of the core protein-coated viral genome to the nucleus [45].

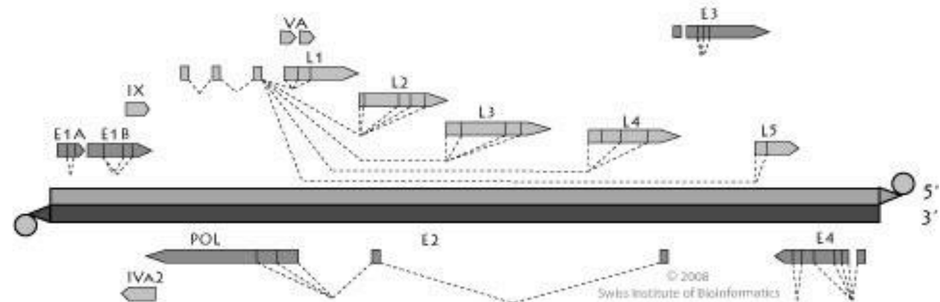

Figure 1.3: Human Adenovirus type 5 genome.

(Source: ViralZone:www.expasy.org/viralzone, Swiss Institute of Bioinformatics)

The structural organization of the AdV genome is depicted in (**Figure 1.3**). The first Ad gene to be expressed is the immediate early E1A gene encoding a transactivator for the transcription of the early genes E1B, E2A, E2B, E3 and E4, as well as protein functions involved in cellular transformation, together with an E1B protein. The E2 region encodes for proteins required for viral DNA replication: DNA polymerase, DNA-binding protein and the precursor of the terminal protein. Replication of the viral genome, which depends on ''inverted terminal repeats'' (ITRs) of 100–140 bp in length at both ends as cis-acting elements (origin of replication) and one copy of the terminal protein (TP) covalently attached to each 5' end as initiation primer, starts about 5–6 h after infection. Core and capsid proteins and a cysteine protease important for proteolytic trimming of TP and other structural proteins are expressed from a common major late promoter after sophisticated splicing of long precursor transcripts. Intranuclear virion assembly starts about 8 h after infection and leads to the production of 10^4 to 10^5 progeny particles per cell which can be released after final proteolytic maturation by cell lysis 30–40 h post-infection, completing the viral life cycle [45].

3.2. Adenovirus vectors

Recombinant AdV vectors provide a highly versatile system for mammalian gene transfer and are widely used in vaccine development and in a variety of gene therapy applications, particularly for gene-based therapy of cancer, including immunotherapy (**Table 1.6**).

Table 1.6: Applications of Adenovirus vectors.

Gene therapy in the treatment of cancer (i) tumour suppressors (ii) oncolytic and sensitizing drug therapy (iii) vaccines
Gene therapy for genetic diseases e.g. Cystic fibrosis
Supplementary therapy e.g. neurodegenerative diseases such as Parkinson's
Production of proteins
Adenovirus recombinant vaccines

These applications are facilitated by several important advantages of AdV over other vectors, including (1) convenient and simple methods of vector construction, (2) efficient transduction of proliferating and quiescent cells, (3) efficient production to high yields, in well-defined cell systems, and (4) high stability, allowing purification and long-term storage (**Table 1.4**, page 12).

The human adenovirus type 2 and 5 are the most used vector backbones for adenovirus-mediated gene transfer. Foreign DNA can be inserted in at least three regions of the adenoviral genome: E1, E3 and the short region between E4 and end of genome. The first generation of adenoviral vectors has deleted E1 and/or E3 regions, which severely impaired their ability to replicate. Deletion of the E1 region impairs viral replication while deletion of the E3 region provides additional free space. Even in the absence of E1 genes first generation AdV vectors are still able to replicate at very low levels, thereby inducing a cellular immune response which is the major impediment to the use of these vectors. Even though anti-AdV antibodies existing in most humans pose an obstacle to readministration of AdV, these vectors can be used for application

where short-term gene expression is required, such as in cancer gene therapy and vaccination. The second and third generations of AdV vectors have deleted E1, E2 and E4 coding sequences [46]. They are less immunogenic than the first generation vectors and provide a larger capacity for transgene insertion [47] [48] [49]. Fourth generation AdV vectors, also called “high capacity” or “gutless” vectors contain only ITR repeats and a packaging signal and can accommodate up to 37 kb of foreign DNA [50, 51]. After entering the nucleus, AdV DNA persists episomally without inserting into host genome [51], thus providing only transient expression of the transgene. Such expression is nonetheless adequate for cancer gene therapy, where the final goal is to kill the tumor cell [46].

Currently, AdV vectors are being tested as subunit vaccine systems for numerous infectious agents ranging from malaria to HIV-1 (**Table 1.7**). Additionally, they are being explored as vaccines against a multitude of tumor-associated antigens. They are attractive vaccine vectors as they induce both innate and adaptive immune responses in mammalian hosts.

Table 1.7: Adenoviral vector vaccines in development for human and veterinary use.

Human	Ref.	Veterinary	Ref.
Plasmodium falciparum	[52]	Avian Influenza virus	[53]
Mycobacterium Tuberculosis	[54]	Mycobacterium Tuberculosis	[55]
Influenza virus	[56]	Foot-and-mouth disease virus	[57]
HIV-1	[58]	PPR virus	[59]
Hepatitis C virus	[60]	Rabbit Hemorrhagic Disease virus	[61]
Measles virus	[62]	Classical Swine Fever virus	[63]
Seoul virus	[64]	Infectious Bronchitis virus	[65]
Rift Valley Fever virus	[66]	Rabies virus	[67]
Japanese Encephalitis virus	[68]	Rift Valley Fever virus	[66]
Cancer	[69-72]	Japanese Encephalitis virus	[68]

4. Peste des petits ruminants

Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease affecting goats, sheep and wild ruminants. It is characterized by high fever, ocular and nasal discharges, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhea [73]. Morbidity and mortality rates vary but can be as high as 100 and 90%, respectively.

The disease caused by PPR virus (PPRV), commonly referred to as ‘‘PPR’’, was first reported from West Africa in the early 1940s and was later (1962) found in Senegal and subsequently recognized as being endemic in west and central Africa (1981). It has also been reported in Sudan (1984) and in east Africa in Kenya and Uganda (1995) and in Ethiopia (1994). PPR was first reported in 1987 in southern India, where it caused epidemics for several years without apparent further spread. The Arabian Peninsula, the Middle East and the remaining parts of the Indian sub-continent were swept by an epidemic of PPR in 1993–1995. Since then the disease has remained endemic in most of these regions and on much of the Indian sub-continent [74]. **Figure 1.4** shows the current known distribution of PPRV.

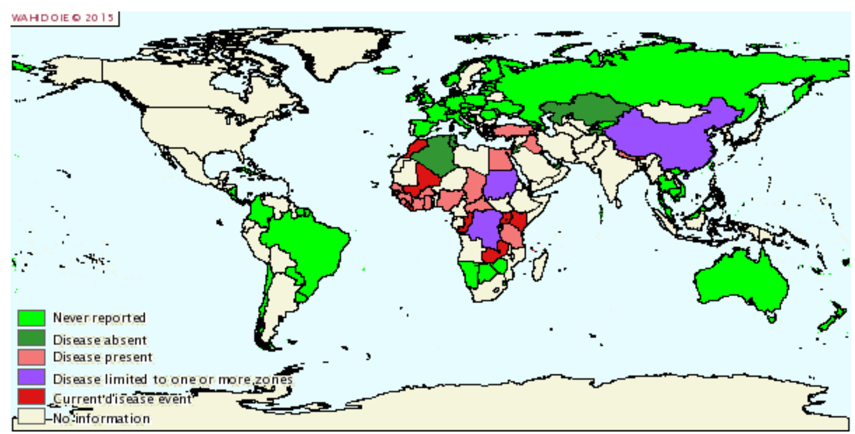

Figure 1.4: Peste des Petits Ruminants distribution map for Jan-Jul 2015.

(Source: World Organization for Animal Health, http://www.oie.int/)

4.1. Peste des Petits Ruminants Virus Biology

PPRV is classified in the *Morbillivirus* genus from the *Paramixoviridae* family along with other important animal and human pathogens like rinderpest

virus (RPV), measles virus (MV), canine distemper virus (CDV), phocine distemper virus (PDV) and dolphin morbillivirus (DMV) [73, 75].

PPRV virions, as the other morbilliviruses, are enveloped particles containing a single strand RNA of negative polarity (**Figure 1.5**). The genome is composed of 15,948 nucleotides. Most viruses in this genus are spherical, and their sizes range from 150 to 300 nm in diameter, but they can be pleomorphic.

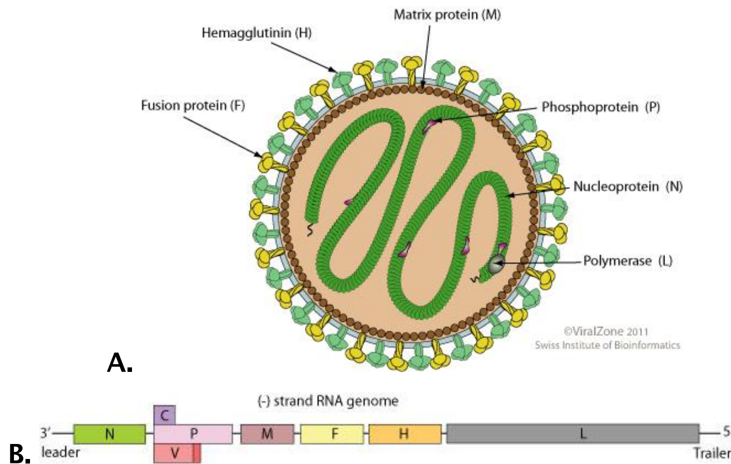

Figure 1.5: Morbillivirus molecular biology. A. Virion; B. Genome.
(Source: *ViralZone:www.expasy.org/viralzone, Swiss Institute of Bioinformatics*)

This genomic RNA is wrapped by the nucleoprotein (N) to form the nucleocapsid into which are associated two other viral proteins: the phosphoprotein (P) and the large protein (L). The phosphoprotein is the co-factor of L, the viral RNA dependant RNA polymerase (RdRp). To the viral envelope which derives from the host cell membrane are associated three viral proteins: the matrix protein (M) which is located inside the envelope and serves as a link between the nucleocapsid and the two external viral proteins, the fusion protein (F) and the haemagglutinin (H). By this position, M plays an important role in ensuring efficient incorporation of nucleocapsids into virions during the virus budding process. The haemagglutinin allows the virus to bind to the cell receptor during the first step of the viral infection process. This binding is followed by the fusion of the viral envelope with cell membrane, a process mediated by F and this leads to the delivery of the nucleocapsids into the cell cytoplasm were takes place the viral multiplication. By their positions

and their functions, both F and H are very important for the induction of protective host immune response against the virus and most of the neutralizing antibodies are directed against H. However N, the most abundant and also the most immunogenic among PPRV proteins, does not induce protective immunity against the virus [76].

PPRV isolates can be grouped into four distinct lineages on the basis of partial sequence analysis of the fusion (F) protein gene. Lineages 1 and 2 are found exclusively in West Africa while lineage 3 has been found in eastern Africa (Ethiopia), Arabia (Oman, Yemen) and in southern India (Tamil Nadu). The fourth lineage is confined to exclusively the Middle East, Arabia and the Indian sub-continent [77].

4.2. Peste des Petits Ruminats Vaccines

Vaccination against PPR is an effective means of controlling the disease. Heterologous rinderpest vaccine was used for many years to control PPR disease. However, after the eradication of rinderpest, it was necessary to restrict the use of this vaccine.

The first attenuated vaccine developed against PPR involved using the lineage I African isolate, Nigeria 75/1 through serial passages on Vero cells [78]. Several trials have demonstrated the efficacy of this vaccine on more than 98,000 sheep and goats in the field between 1989 and 1996. During those trials no unwanted side effects such as abortion in pregnant animals were recorded. It was demonstrated also that animals vaccinated with this attenuated PPRV were unable to transmit the challenge virus to in-contact animals. Anti-PPRV antibodies generated by vaccinated animals last for at least 3 years, the effective economic life of the animals. PPRV Nigeria 75/1 belongs to lineage I. During the development process of the attenuated vaccine based on this virus, different PPRV strains were used as challenge viruses and all failed to induce disease in the vaccinated animals, result demonstrating the potential worldwide effective use of this vaccine to control PPR.

Subsequently, three more attenuated vaccines were developed in India by using the lineage IV Indian isolates. Since lineage IV is mostly restricted to Asian countries, the use of Nigeria 75/1 vaccine in Asian countries may increase the likelihood of mixing up of lineages and the development of

mutants with high virulence. Thus, it is imperative to consider using the lineage-specific vaccine available for use in Asian countries. To this effect, a live-attenuated Vero cell-adapted vaccine against PPRV, Sungri/96 vaccine, was developed by the IVRI. The Sungri isolate was adapted in Vero cells and was found to be attenuated fully at 59 passages (P-59). This vaccine has been tested extensively both experimentally as well as in the field and has been found to be safe and potent in small ruminants. The third and fourth conventional live-attenuated vaccines were PPRV Arasur 87 (sheep origin) and Coimbatore 97 (goat origin), respectively, which were developed by TANUVAS. These vaccines are being used in southern states of India. They are also as equally safe and protective as Sugri 96 in sheep and goats and have potential for commercial vaccine production [79].

The attenuated homologous PPRV vaccines are now the only vaccines permitted for use in sheep and goats to protect them against PPRV infections [76]. The PPR available vaccines are summarized in **Table 1.8.**

Nowadays these vaccines are produced in Vero cells using classical techniques, i.e. in T-flasks or roller bottles. These strategies involve high efforts concerning consumables and have limited scalability, significantly increasing the bioprocess costs. Thus there is a need for better vaccine production processes for controlling future PPR outbreaks.

Alternative PPR vaccines are under development to counteract the major disadvantage when using classical live attenuated vaccine is that the antibody responses they induce in animals cannot be distinguished from those following a natural infection (**Table 1.8**).

This makes sero-epidemiosurveillance of the disease impossible in endemic areas where a vaccination programme has been or is being implemented. A way to combine activities, vaccination and serosurveillance, for the better management of the disease would be the use of DIVA vaccines, the acronym used for vaccines which enable differentiation between infected and vaccinated animals. Originally this term was applied to gene-deleted marker vaccines for large DNA viruses when used with their vaccine specific serological tests, but it can also apply to subunit vaccines, heterologous vaccines or some killed whole pathogen vaccines such as the highly purified Foot-and-mouth disease vaccine

which is used in conjunction with non-structural protein-based serological tests. It can be used also for recombinant-based vaccines [76].

Table 1.8: PPR available vaccines.

Producer	Product Name	Type	Strain/ Subtype	Licensed Countries
Botswana Vaccine Institute	PPR-VAC®	Live	Nig 75/1	Botswana
Central Veterinary Control and Research Institute	Freeze Dried PPR Vaccine	Live	Nig 75/1	Turkey
Dollvet	Pestdoll-S	Live	Nig 75/1	Turkey
Intervac (PVT) Ltd.	Pestevac	Live	Nig 75/1	Pakistan
Jordan Bio-Industries Center (JOVAC)	PESTEVAC	Modified Live	Nig 75/1	Afghanistan, Albania, Bahrain, Ethiopia, Iraq, Jordan, Kuwait, Lebanon, Libya, Malaysia, Oman, Pakistan, Syria, United Arab Emirates, Yemen
National Veterinary Institute	Peste des Petits Ruminants Vaccine	Live	Nig 75/1	Ethiopia
Nepal Directorate of Animal Health	Peste des Petits Ruminants Vaccine	Live	Nig 75/1	Nepal
Vetal Company	Pestvac K™	Live	Nig 75/1	Turkey
Indian Veterinary Research Institute (IVRI)	PPRV Sungri/96	Live	Sungri 96	India
Tamil Nadu Veterinary and Animal Sciences University (TANUVAS)	TN MIB /87	Live	Arasur 87	India
	PPR Vaccine - TN/97	Live	Coimbatore 97	India
Veterinary Serum and Vaccine Research Institute	PPR-TC Vaccine Attenuated	Live	Egypt 87	Egypt
Merck Animal Health	OVILIS® PPR	Live	Sungri/96	n.a.
Hester Biosciences Limited	PPR Vaccine - Sungri 96	Live	Sungri 96	India
	PPR Vaccine - Nigerian 75/1	Live	Nig 75/1	India
Indian Immunologicals Limited	Raksha PPR	Live	Sungri 96	India
National Veterinary Research Institute	Peste des Petits Ruminants Virus Vaccine	Live	PPR V 75/1	Nigeria

(Sources: The Center for Food Security and Public Health, http://www.cfsph.iastate.edu ; MSD Animal Health, http://www.merck-animal-health.com; [80])

Table 1.9: PPR new candidate vaccine types.

Vaccine Type	Characteristics	Ref.
Viral vector		
Recombinant vaccinia virus (rVV)	rVV simultaneously expressing F and H proteins of rinderpest virus (RPV)	[81]
Modified Vaccinia virus Ankara virus (MVA)	MVA expressing either H or F protein of PPRV	[82]
Recombinant capripoxvirus (rCPV)	rCPV expressing either F or H protein of RPV	[83]
	rCPV expressing H protein of PPRV	[84]
	rCPV expressing F protein of PPRV	[85]
	rCPV expressing either H or F protein of PPRV	[86]
	rCPV expressing either H or F protein of PPRV	[87]
Recombinant canine adenovirus type-2 (rCAV-2)	rCAV-2 expressing H protein of PPRV	[59]
Recombinant human adenovirus type-5 (rHAV-5)	rHAV-5 expressing either F or H protein of PPRV	[88, 89]
Recombinant adenovirus (rAdV)	rAdV expressing H and F proteins of PPRV	[90]
	rAdV expressing H protein of PPRV	[91]
Chimeric virus		
Chimeric baculovirus (BV)	Chimeric BV carrying a membrane bound form of H protein of PPRV	[92, 93]
	Chimeric BV carrying immunodominant ectodomains of F protein of PPRV	[94]
Reverse genetics		
Recombinant peste des petits ruminants virus (rPPRV)	rPPRV expressing green fluorescent protein (GFP)	[95]
	rPPRV expressing foot-and-mouth disease virus (FMDV) VP1	[96]
Subunit		
H protein subunit vaccine	H protein of PPRV expressed in transgenic peanut plants	[97]
Virus-like Particle (VLP)	VLP composed of PPRV M and H (or F) proteins	[98]
Nucleic acid		
Suicidal DNA vaccine	Recombinant pSCA1 plasmid expressing H protein of PPRV	[99]

(Source: Adapted from: [100])

Following the DIVA approach, the F protein of PPRV was inserted into the genome of an attenuated capripox virus vaccine [85]. The resulting recombinant virus expressed the PPRV F protein on the surface of infected cells, which was recognized by an anti-F monoclonal antibody. This recombinant was then tested in goats and shown to be effective in protecting inoculated animals against PPR at a dose as low as 0.1 pfu. A similar vaccine

which expressed the PPRV H protein was also produced and it is effective at a minimal dose of 10 pfu [84]. Another recombinant PPRV vaccine has the rinderpest vaccine virus genome as the backbone into which the matrix (M), the F and H protein genes of RPV were replaced by those of PPRV. The resulting chimeric virus proved to be a safe and effective vaccine which could protect goats against virulent challenge with PPRV [101]. A recombinant Canine Adenovirus type 2 expressing the H gene of PPRV was generated in MDCK transfected and used to immunize goats. All vaccinated animals produced antibodies upon primary injection that were effective in neutralizing PPRV in vitro [59].

5. Virus production process

A virus production process compromises four main steps (**Figure 1.6**). Since viruses only grow within living cells, viruses for therapeutic applications are propagated in cells (e.g. in chicken eggs) or in continuous cell lines (e.g. Vero cells). Once the virus has been propagated, it must then be isolated from the cells and the cell-culture medium. This may be achieved by several techniques including chemical lyses of the cell, centrifugation and filtration, or homogenization.

Figure 1.6: The four steps in viral production.

The next step, purifying the virus, may likewise involve multiple techniques of centrifugation, ultra-filtration or chromatography, or chemical purification. At this stage, viruses may also be chemically inactivated for killed vaccine preparations. Then the viral preparation is formulated by mixing it with the constituents that allow each dose to be safely delivered in the right concentration. This is the point where the product may also be combined with other antigens (e.g. combined vaccines like the measles–mumps-rubella vaccine). The formulated product is filled in vials or syringes. Some vaccines or other viral products are freeze-dried (lyophilized) at this stage, to prolong their shelf-life.

5.1. Production

An animal cell-based bioprocess for virus production has the particularity of constituting a system where the cell physiology is often sharply modified and redirected by the virus infection. It differs considerably from a cell-based recombinant protein synthesis that constitutes ultimately a consequence of a stable cell physiological state. As a consequence, parameters for a virus production bioprocess establishment have to be worked out by studying both, the cell growth and the virus replication steps.

The production of viruses encompasses several bioengineering challenges (**Figure 1.7**) that must be carefully addressed in order to improve up- and downstream processing with the final goal of maximizing performance and reducing production costs. In general, all virus culture processes start by growing the cell line of choice to the desired cell concentration for infection. Then, the infection and virus production cycle is initiated by introducing a virus stock.

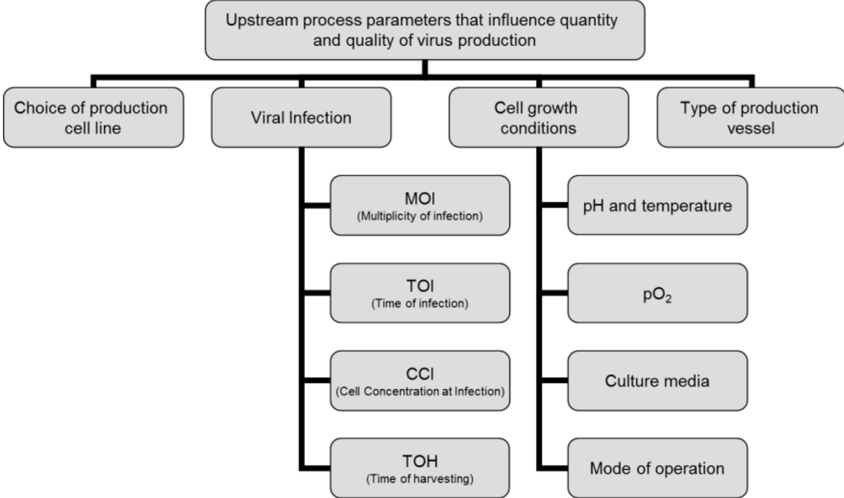

Figure 1.7: Upstream process parameters that influence quality and quantity of virus production. (Source: [102])

In some cases virus production can be obtain, not by infection but by transfection of cells with plasmids containing the all the viral components (e. g. lentivirus [103] or AAV vectors [104]) or by packaging cell lines that constitutively have the viral components introduced in their genome such as

293 FLEX [16] and Flp293A [17] developed for retrovirus production. In here it will be focused mainly in the processes performed by viral infection.

5.1.1. Cell lines for Viral Vaccine production

Virus productions were initially performed using the natural host of the virus. Upon the establishment of cell culture technology in 1950s, animal cell cultures gradually replaced live animals in the preparation of viral antigens for vaccine. The observation by Enders and co-workers [105] that non nervous tissue culture could be used to replicate and produce poliovirus paved the way to large scale production of vaccines. This discovery led to the development of the first commercial product generated using mammalian cell cultures (primary monkey kidney cells), the poliovirus vaccine. Regrettably, primary monkey kidney cells presented many drawbacks such as the relatively high risk of contamination with adventitious agents (contamination by various monkey viruses), shortage of donor animals, use of endangered animals as cell source, use of non or insufficiently characterized cell substrates for virus production, limited expansion and obligatorily adherent cell growth [106-109]. In the 1960s, human diploid fibroblast cells, WI-38 [110] and MRC-5 [111], and baby hamster kidney cells (BHK-21 (C13)) were established and used for the production of a vaccine against rabies virus [112] and foot and mouth disease [113], respectively.

The manufacturing capability for viral vaccines produced in embryonated eggs and conventional/ classical cell substrates, such as chicken embryo fibroblasts, has now reached its capacity limit. This constraint may be overcome by utilizing other recognized cell substrates such as Madin Darby Canine Kidney (MDCK) (dog origin), Chinese Hamster Ovary (CHO) (hamster cells) or Vero cells (monkey origin) or as an alternative, introduce new cell substrates of human or avian origin. Using new cell substrates may prove to be a highly replication-proficient way of producing live viral vaccines such as Influenza A viruses.

The Vero cell line was the first continuous mammalian cell line, established from African green monkeys in 1962, and is currently the most widely accepted

by regulatory authorizes for vaccine development due to the fact that Vero-derived human vaccines have been in use for nearly 30 years (**Table 1.10**).

Table 1.10: Some Human viral licensed vaccines produced in cell lines.

Vaccine name	Manufacture	Tradename	Cell Line for production
Hepatitis A Vaccine, Inactivated	GlaxoSmithKline Biologicals	**Havrix**	MRC-5
Hepatitis A Vaccine, Inactivated	Merck & Co., Inc.	**VAQTA**	MRC-5
H5N1 pandemic influenza vaccine, inactivated	Baxter AG	**Preflucel**	Vero
Japanese Encephalitis Vaccine, Inactivated, Adsorbed	Intercell Biomedical	**IXIARO**	Vero
Measles Virus Vaccine Live	Merck & Co., Inc.	**ATTENUVAX**	chick embryo cell
Measles, Mumps and Rubella Virus Vaccine, Live	Merck & Co., Inc.	**M-M-R II**	chick embryo cell chick embryo cell WI-98
Measles, Mumps, Rubella and Varicella Virus Vaccine Live	Merck & Co., Inc.	**ProQuad**	chick embryo cell chick embryo cell WI-98 MRC-5
Poliovirus Vaccine Inactivate	Sanofi Pasteur, SA	**IPOL**	Vero
Rabies Vaccine	Sanofi Pasteur, SA	**Imovax**	MRC-5
Rotavirus Vaccine, Live Oral	GSK	**Rotarix**	Vero
Rotavirus Vaccine, Live Oral	Merck & Co, Inc.	**RotaTeq**	Vero
Smallpox (Vaccinia) Vaccine, Live	Acambis Inc.	**ACAM2000**	Vero
Varicella Virus Vaccine Live	Merck & Co, Inc.	**Varivax**	MRC-5
Zoster Vaccine, Live	Merck & Co., Inc.	**Zostavax**	MRC-5

(Adapted from: [6])

Currently, there are several human viral vaccines licensed using cell substrates (see **Table 1.10**) or under clinical trials such as the Influenza vaccine produced using Vero [114-116], MDCK [117, 118] or PER.C6 [119, 120] cells amongst others [121]. Despite these significant advances in vaccine manufacturing, there are vaccines still produced in eggs such as the recently approved Influenza A (H1N1) 2009 monovalent vaccines from CSL, Medimmune LLC or Novartis Vaccines and Diagnostics.

Novel cell substrates originate predominantly from avian and human (or other mammal sources) and the latter include tree human cell substrates

HEK293 cells, PER.C6 cells and CAP cells. These human cell lines were developed by transforming or stably transfecting human embryonic kidney cells (HEK293), human embryonic retinal cells (PER.C6), and primary human amniocytes (CAP, from CEVEC) with the early region 1 (E1) of adenovirus type 5 (AdV5) [122-125]. Due to its glycosylation machinery, human cell lines have also emerged as a new and powerful alternative for the production of human therapeutic proteins. Biopharmaceutical companies and researchers are evaluating the best expression system to produce human recombinant proteins and today most companies are considering the clinical, quality, and productivity implications of their expression system selections.

Nowadays, additional novel cell substrate of avian origin are available such as the duck embryonic stem cell line EB66 [126] or the CR (Cairina Retina) cell line derived from muscovy duck retinal tissue. The CR cell line was subsequently transfected with AdV5 E1-genes and AdV5 pIX gene resulting in AGE1.CR.pIX. AGE1.CR.pIX also supports replication of the Vaccinia Virus MVA85A, giving rise to high yield of this increasingly popular live attenuated vaccine strain [127, 128].

5.1.2. Cell lines for Adenovirus production

The most common and well documented packaging cell line for adenovirus production is the HEK293 cell line, which contains the E1 region of the adenovirus [122]. Homologous recombination between the left terminus of first-generation adenovirus vector or helper-virus DNA and partially overlapping E1 sequences in the genome of HEK293 cells normally lead to the generation of replicative competent adenoviruses (RCA) [129]. The presence of RCA is clearly undesirable as they may replicate in an uncontrolled manner. In recombinant adenovirus batches to be used in human patients, RCA is potentially hazardous, especially in immuno-compromised patients, being associated to inflammatory responses [130]. The FDA guidelines demand the presence of less than 1 RCA in $3x10^{10}$ vector particles for clinical applications (Biological Response Modifiers Advisory Committee, 2001).

Alternative host cell lines have been developed to overcome this problem either by reducing the overlapping sequences or by eliminating any overlap, as is the case of N52.E6 cells [123] and PER.C6 cells [125]. The PER.C6 cell line,

derived from human embryonic retinal cells, licensed by Crucell, The Netherlands, has been established for industrial applications (e.g. full traceability available) due to its reduced propensity to generate RCA and its capacity to achieve high yields of adenovirus vectors. Much of the industrial development of PER.C6 has been driven by collaboration between Crucell and Merck & Co. for the generation of an Ad vector-based vaccine against HIV infection. Due to commercial reasons the PER.C6 cell line is currently not freely available to the research community.

Human amniocytes were identified as an alternative cell source for the reproducible generation of stable cell lines following transformation with E1 functions [123] and E1/pIX genes [124]. The design of the transforming plasmid construct in one of the cell lines (N52.E6) was similar as in PER.C6 cells [123], in principle excluding the generation of RCA due to the absence of any sequence overlap between vector DNA and the integrated E1 region. Production yields of AdV vectors in N52.E6 cells have been high. Because of legal reasons this cell line is currently not distributed.

There have been additional attempts to generate production cell lines for AdV vectors. Unlike the cell lines discussed above, they all were based on established cell lines such as HeLa and A549 cells [131-134]. To our knowledge these cell lines have not been distributed broadly. One of the cell lines, developed by Farson and colleagues [134], was generated by retrovirus-mediated gene transfer without antibiotic selection, having the E1A and E1B expression constructs on different retroviral vectors. In principle, this design could reduce the generation of HDEPs that have been observed in PER.C6. In a similar approach, E1A and E1B functions were consecutively introduced by plasmid transfection/selection into A549 cells [135]. To prevent E1A-mediated apoptosis the E1A construct contained small deletions within the E1A coding region resulting in the removal of amino acids 4 to 25 and 111 to 123 from the E1A proteins. In A549 cells expressing this mutant form of E1A, expression of the E1B 55K protein was sufficient for efficient transcomplementation of AdV vectors while expression of the E1B 19K protein inhibiting E1A-induced apoptosis was not required. Moreover, RCA was not observed during production.

Another disadvantage of human adenoviral vectors is their limited clinical use; 90% of the population has developed pre-existing humoral and cellular immunity to those vectors [18]. Sustainable platforms for the generation of vectors from different human serotypes or derived from non-human adenovirus at similar or greater titers than those obtained with human adenovirus vectors and free of detectable levels of RCA are required [20, 21]. One example is the production of canine adenovirus type 2 (CAV-2) vectors in dog kidney cells [18, 19]. With these vectors, the risks associated with RCAs are diminished, if not completely eliminated, because CAV-2 does not propagate in human cells; also, CAV-2 transduces human-derived cells at an efficiency similar to that of human adenovirus type 5, and are amenable to *in vivo* use [18]. Novel cell lines for the production of these CAV-2 vectors are under evaluation, with emphasis in MDCK cells [136, 137].

5.1.3. Cell growth conditions and viral infection

Production methods differ according to the cell type used: adherent or suspension cell culture. Adherent cell lines have shown a higher cell specific productivity compared to suspension-adapted cell lines [138]. Nevertheless, surface requirements for cell growth make these processes difficult to scale up to large volumes and high cell densities. The use of roller bottles and cell factory systems for cell cultivation offer a large surface for cell growth. Scale-up is linear and up to 10 cell factories have been used to produce AdV within the HEK293 cell line. Specific productivity was maintained high with active gassing and 8×10^3 infectious particles (ip)/cell were achieved [139]. Roller bottles have been also used for the cultivation of Vero cells and the production of rabies virus [140] and PPR virus [141] amongst others.

A great step towards the establishment of high-concentration cell cultures and industrial bioprocesses was done by the cell cultivation on suspended beads, named microcarriers. Batches of 6000L Vero cell cultures [142] on microcarriers can be performed in bioreactors in serum-free medium, with no loss in productivity, and have been used for the production of vaccines as rabies and poliomyelitis [114]. Several types of microcarriers have been

evaluated for cell culture and virus production, from macroporous [143] to solid matrices [144, 145].

Microcarriers also offer a better scalable solution to for the production of AdV with adherent cell lines; with Cytodex-1 and Cytodex-3 microcarriers up to 1.5×10^4 ip/cell have been produced in spinner flasks whereas 2×10^4 ip/cell have been produced in small-scale static cultures [146]. Iyer et al. have demonstrated that specific productivity was similar using Cytodex-3 microcarriers in serum containing cultures and in suspension serum-free culture in a bioreactor [138]. However, recovery of virus from the cells attached to microcarriers is cumbersome, requiring cell detachment from the microcarriers by trypsinization.

Suspension cell lines are better suitable for large scale production since they avoid the use of microcarriers facilitating viral production processes (**Table 1.11**).

Table 1.11: Engineering Challenges for Cell Cultivation Methods Microcarriers Free Cell Suspension.

Microcarriers	Single Cell Suspension
Anchorage dependent cells. Cell expansion often occurs in roller-flasks prior to bioreactor.	Cells grow in single cell suspension. Culture is homogenous and sampling is easy. Process is simple, scalable and robust. No additional factors or proteins are necessary for adhesion or expansion. Costs are lower.
Major Challenges	Major Challenges
• Shear stress and mixing adaptation of stirrer speed for attachment. • Sub-passages. • Longer lag phase. • Microcarrier preparation (swelling and autoclaving). • Trypsin/or other detachment agent for cell-passaging.	• Increase volume. • Lengthen cell-culture lead time.

(adapted from [147])

HEK293 cells have been adapted to suspension and are widely used for the production of AdV [102], lentivirus [103], adeno-associated virus [104] and have been also evaluated for the production of influenza virus [148]. PER.C6 cells were also exploited for the manufacture of classical vaccines including influenza and West Nilo virus, besides their use in AdV production.

Recently, Vero cells [149] and MDCK cells [150] have been able to adapt to suspension which constitute valuable information for the development of a low-cost high-productivity process using a suspension culture of Vero cells to produce viral vaccines.

Different operation modes have been developed to achieve high titer yields for virus production: (i) the batch mode provides the easiest approach as no extra feeding is required and the risk of contamination is lower, given the simplicity of operation; (ii) the fed-batch mode is easy to operate and readily scalable; it is primarily used to extend culture lifetime by supplementing limiting nutrients and/or reducing the accumulation of toxic metabolites; and (iii) the perfusion mode consists in retaining the cells leading to a higher cell concentration inside the bioreactor, while fresh medium is supplied, allowing replenishing of nutrients and removal of toxic metabolites (**Figure 1. 8**).

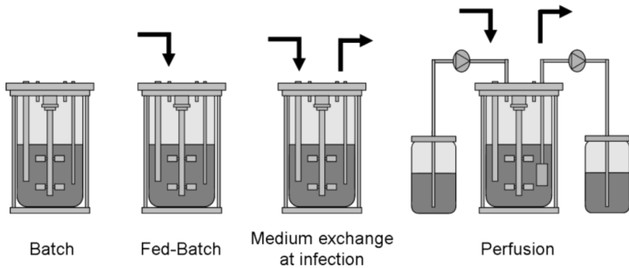

Figure 1. 8: Modes of culture operation. Source: ([151])

The optimization of culture media for animal cell cultures, has received a lot of interest for many years owing to its critical impact on the development of bioprocesses based on animal cell technology. Many attempts have been made to replace serum and to design serum-free media [138, 152-155]. Serum contains thousands of beneficial components, including growth factors, cytokines, hormones, attachment factors, essential nutrients such as trace elements and other unknown components promoting cell survival and proliferation in vitro. Nevertheless, the use of animal sera shows many shortcomings such as the potential introduction of animal-derived proteins and contaminants (e.g., fungi, bacteria, viruses, or Bovine Spongiform Encephalopathy (BSE) agent), a non-defined composition, a variable quality and a high cost. Moreover, the presence of serum in culture medium complicates

downstream processing. Various formulations of serum-free and animal/human protein-free media are commercially available for the cultivation of several cell lines.

Large-scale manufacturing processes typically use fully controlled stirred tank bioreactors, which allow not only monitoring but also controlling several the culture parameters like pH, DO and temperature, for both microcarrier and suspension culture. Overall, the ideal production process for a therapeutic product should meet several acceptance criteria: i) use of safe cell lines; ii) use of animal component-free culture medium and iii) rapid production process that yields high concentrations of the product in a cost effective way. The expected virus productivity (cell-specific and volumetric) depends on the overall virus and the cell line used to propagate it.

New trends for the use disposable culture-ware like Wave® [156] show numerous advantages for GMP production, such as avoiding the need for validated cleaning and sterilization. This makes this type of bioreactor the method of choice, providing that a manageable number can be used to produce the desired amount of viral particles. But scaling vaccine production requires a significant investment in time and money. Even for relatively simple processes, like vaccine packaging, up to 2 years may be required to install and validate new packaging machinery. Building a new manufacturing facility takes on average 5 years to complete and validate with regulatory authorities.

5.1.3.1. Infection

Process-related parameters such as the multiplicity and time of infection (MOI and TOI, respectively), time of harvest (TOH) and cell concentration at infection (CCI) strongly influence virus productivities. Adequate MOI, defined as the number of virus particles *per* cell at the time of infection, is determinant for attaining optimal yields; the lowest possible MOI is desired to avoid rapid depletion of costly and certified master virus banks and to ensure that the infection kinetics is reproducible between different production scales.

The optimum MOI is dependent on the virus seed properties and virus construct. The seed properties to consider include: the ratio of total to infectious particles (tp/ip ratio), the stability of the virus during storage and handling, and the stability of the virus during the infection process. In addition,

the measurement of virus particles is defined by the specific assay format and the specific standard used. Different laboratories use different methods, which frequently make the direct comparison of MOIs from one system to the other difficult or even impossible. The optimal MOI is determined for each system to ensure that the cells are infected with an adequate number of viral particles for maximum volumetric virus productivity.

Because AdV infections are rapid (a regular infectious cycle takes about 48 hours) and lytic, processes are usually configured as single-round infections with MOI >1 to ensure all that cells are infected. For AdV cultivation processes, a MOI of 5-200 virus particles/cell (vp/cell) is typically used, resulting in an amplification ratio usually higher than 200 in HEK293 [157, 158] and PER.C6 cells [159, 160]. On the other end enveloped virus have different infection cycles, (released by budding from the cells), the MOI used for this type of virus are inferior, normally lower than 1, which takes several cycles of virus attachment, replication, and release before all cells in a culture are infected. In vaccine production processes at large scale the use of low MOI avoid the preparation and handling of large volumes of virus seeds. For influenza virus production in Vero and MDCK cells it is typically used a MOI of 0.001-0.5 [161-163]. The same range of MOIs are also used for the production of Rabies virus in Vero cells [164] and BHK cells [165], the production of Japanese encephalitis virus [166], parainfluenza virus type 3 (PIV3) and respiratory syncytial virus [167], among others. Recently, Yamada et al. reported the production of AdV with a very low MOI (0.001), however this requires several medium exchanges and the vectors are only collected 10 days-post infection [168].

The harvest time depends on several factors, such as the virus itself and the cell line used. A medium that will sustain cell viability for longer periods will also improve the final titers. For lytic virus like AdV, because the titer is maximal when cell viability is still high, the time of harvest is generally in the range of 48-72 hours post infection (hpi). At this point the cells are harvested and further lyzed by freeze-thaw or by use of a detergent. In this way AdV has been preferably recovered in the intracellular fraction thus allowing a reduction of volumes in the downstream processing. For viral vaccine productions, with low MOIs, the harvest time is normally much longer ranging from 96 hours to more than 2 weeks depending on cell line and culture process used [169, 170].

The virus then mainly recovered in the supernatant leading to high volumes to be handled in downstream.

For production of influenza virus, rabies virus and others, in Vero, MDCK and other cells adherent cultures, several strategies have been applied to increase the cell concentration for infection and consequently virus productivity. The increasing the concentration of microcarriers [171], changing the type of microcarriers [143], change the culture medium for infection [154], alteration of the culture mode [172, 173], were some of the strategies applied. The cell culture in serum-free and serum containing medium have been also evaluated on cell growth and viral productivity [152, 163, 167, 169]. Production of influenza virus in 1,200 L was possible with $5x10^6$ Vero cells in stirred tank bioreactor with Cytodex-3 microcarriers [161]. It has been showed that viral productivity was dependent upon the metabolic state of the cells rather than the phase of growth at which the cells were infected [174].

Despite the fact that medium formulations, that allow maximum cell growth up to $3–9x10^6$ cell/mL for HEK293 and PER.C6, in serum-free media are available [157, 175, 176], optimal AdV production titers in batch culture cannot be achieved beyond a CCI of $5x10^5$ cell/mL. This is often referred to as the ‘‘cell density effect’’. Garnier et al. demonstrated that growing cells to $0.3-0.5x10^6$ cell/mL and infecting the cells will result in a good productivity [177]. Infection at $1x10^6$ cell/mL requires media exchange at the time of infection in order to maintain a similar specific yield. By implementing a media exchange at the time of infection, Zhang et al. (2001) have reported one log increase in their final volumetric productivity (pfu/mL) at day 6 in the supernatant [178], at a cell concentration of $1.5x10^6$ cell/mL. Liu and Shoupeng (2001) showed an improved infection when the cells are between 40% and 70% of cell maintenance phase [179]. Also the authors mention the importance of changing at least 90% of the medium at the infection time. It is generally accepted that infecting cells at $1x10^6$ cell/mL is the optimal cell concentration after medium renewal. Studies on the cell environment have permitted to enhance specific AdV productivity through better cell culture environment control (medium formulation and feeding strategies). These strategies have also been developed to increase the cell concentration at infection to ultimately enhance volumetric productivity. The challenge has been to keep the specific

productivity obtained at low cell concentration constant without feeding. The cell density effect describes a drop in the specific virus production when the cell concentration is above 0.5×10^{6} cell/mL [180].

The major hypothesis behind the cell density effect is that nutrient depletion and metabolite accumulation hinders cell metabolism and renders them unable to support viral production at high cell densities. Consequently, in order to improve substrate renewal and metabolite removal, production modes have gained in complexity from batch to perfusion system. Fed-batch strategies have failed to maintain a high specific productivity above 2×10^{6} cell/mL [177]. High cell concentration at high viability could only be attained using perfusion systems through a continuous renewal of medium. Compared to a batch cell culture infected at 0.5×10^{6} cell/mL with medium replacement, specific productivity has been maintained at 1.8×10^{4} virus vp/cell when infection was performed on a 3 L perfusion cell culture at ~3×10^{6} cell/mL, with 2–3 volumes/day under serum-free conditions [181]. Up to 4.1×10^{10} vp/mL has been produced under these conditions. Cortin et al. (2004) have infected 8×10^{6} cell/mL at 35 °C at 1 volume/day in 5% serum containing medium to produce ~8×10^{9} ip/mL [182]. Production of replicating AdV vectors in HeLa cells has been successfully performed at infection densities of 5 to 14.6×10^{6} cell/mL at 1.5–2 volumes/day, under serum-free conditions [182]. Productivities have reached 3–6×10^{11} vp/mL and 3–5×10^{4} vp/cell [183].

Another avenue to explain the cell density effect has been studied by Zhang and co-workers [184]. They demonstrated that a decrease in the proportion of cells in S phase was also responsible for a decrease in specific productivity at high cell densities. Ferreira et al. chemically synchronized HEK293 cells at each phase of the cell cycle and a 2.6-fold increase in AdV cell specific titer was obtained when the percentage of cells at the S phase of the cell cycle was increased from 36% to 47% [185]. S phase synchronization was also obtained by decreasing the culture temperature to 31ºC during 67 hours and restoring it to 37ºC during 72 hours. By using this strategy they were able to synchronize 57% of the population in the S phase of the cell cycle obtaining an increase of 7.3-fold on AdV cell specific titer after infection.

Besides the cell cycle phase of the cell, the specific metabolic requirements for both cell growth and virus production have been analyzed. Analysis of

central cell metabolism has permitted to identify differences in consumption and production rates during growth and infection [160, 186]. A favorable metabolic state for AdV production has been defined by an increase in glycolytic fluxes, in TCA fluxes and in ATP production rates upon infection [187, 188]. This state might be prolonged at higher cell densities if the feed rate is adjusted [188]. In better medium/feeding strategies, glucose enters more efficiently into the TCA cycle and glutaminolysis and amino-acid catabolism rates are reduced. Lower lactate and ammonia production rates have been consequently observed [188, 189]. Metabolic flux analysis is therefore useful to determine culture conditions resulting in enhanced productivity. It provides a basis for a rational approach to improve medium and feeding strategies, to design better cell lines with improved TCA activity and ATP production rates and to implement on-line control of such fluxes.

5.1.3.2. Physical parameters

Physical parameters such temperature, pH, dissolved oxygen (DO) and partial CO_2 pressure should also be studied and optimized for each virus cultivation system.

The optimum virus cultivation temperature can be different from the optimum cell growth temperature (37°C) and should be optimized. The decrease of temperature during virus infection has been applied for several virus production processes, mainly due to lower viral stability. Jardon and Garnier (2003) reported significantly increased virus productivity at 35°C compared to 37°C for the cultivation of AdV in HEK293 cells and temperature affecting also the cellular metabolism and the rate of cell death post-infection [190]. Several virus processes with Vero cells have been performed with shift to lower temperatures post-infection (35-33°C) to increase viral yields, namely hepatitis A virus [191], attenuated chimeric virus of parainfluenza virus type 3 and respiratory syncytial virus [167]. However, some times the decrease of temperature has a detrimental effect on the type of virus produce, as in the case of retrovirus were the virus produced at 32°C were more unstable than those produced at 37°C due to changes in the cholesterol content in the viral membrane [192].

The pH is another parameter that needs to be taking into account for cell growth and viral infection. For the production of Rabies virus in Vero cells for example, the pH used for cell culture (7.2) is changed to 7.4 during viral infection phase [193]. For the production attenuated chimeric virus of parainfluenza virus type 3 and respiratory syncytial virus, also in Vero cells, it was showed that cell growth and virus production were pH-independent at in the pH range of 7.0-7.4 [167]. Retrovirus production in the range 6.0 to 7.8 of pH levels revealed a significant decrease in virus titers at levels below 6.8 and above 7.2, optimum titers being achieved in cultures at pH 7.2 [194]. The pH effect on AdV production was evaluated in HEK293 cells showing no virus production at pH 6.7 and 7.7 but only at 7.2, with no effect on cell growth observed [190]. Xie et al. using PER.C6 cells found the optimal pH for AdV production to be 7.3 [195]. They observed under different pH conditions (6.8–7.6) that cell metabolism in both infected and non-infected cultures was dramatically changed leading to significant differences in glucose/glutamine consumption and lactate/ammonium production. Ferreira et al. studied effect of ammonia accumulation on intracellular pH (pHi) and its consequent effect on AdV production showing that AdV volumetric productivities were lower when the cells were exposed to the lower pH (pHi 7.0) [196].

There are only few published reports addressing the effects of other process parameters such as DO and pCO_2 on virus cultivation. In most controlled processes for AdV and other virus, the DO is usually set at greater than 30% of air saturation [197]. For insect cells and VLP production with baculovirus it has been showed that, although the DO did not show a significant effect upon cell growth in the range from 10 to 50%, cell infection and specific productivity were dramatically affected. The production was optimal at a DO of 25% with decreases higher than 50% being observed when the DO decreased to 10 or increased to 50% [198]. For the production of Rabies virus or Hepatits A virus in Vero cells it has been used a DO for cell growth (40-50%) and then a reduction of that value to (25-30%) during cell infection phase [191]. It has been reported for Vesicular Stomatis virus (VSV) production in Vero cells a decrease in viral productivity with an increase of the oxygen partial pressure due to an increase of the reactive oxygen species that increase the oxidation of the viral proteins [199]. Dissolved oxygen levels in the range 20-

80% in retrovirus production experiments had no significant effect on titers [194].

It has been showed In most cases pCO_2 is not controlled and sometimes not even monitored, although Jardon and Garnier report a decrease in virus productivity with increased pCO_2 [190].

Shear stress can also be an important variable for bioreactor operation due to its influence on cell growth and product expression. Owing to the lack of cell walls, animal cells are highly sensitive to hydrodynamic shear stress typically generated in stirred-tank bioreactor vessels (i.e. agitation mixing and bubble sparging). Cell injuries due to fluid-mechanical forces arise mainly from either the shear stress generated by hydrodynamic mixing or the cell-bubble interactions generated by air sparging. The hydrodynamic shear stress could inhibit culturing of anchorange-dependent cells on microcarriers [200], since the cells adhering to microcarrier surface are less robust to disperse fluid-mechanical forces than the freely-suspended single cells.

5.1.3.3. <u>Process monitoring</u>

The success of a bio-product requires a controlled and monitored process and a well characterized product. For virus quantification, several assays have been developed such as plaque-forming unit (pfu) and tissue culture infectious dose 50 ($TCID_{50}$) assays to determine the number of infectious virus particles, and optical absorbance measurement or electron microscopy (EM) to determine the number of total particles [201]. Other methods for quantification are based on flow cytometry [202], slot-blot analysis [203], real-time Q-PCR [204, 205], and high performance liquid chromatography (HPLC) [206, 207], only some of which are suitable for in-process monitoring [208]. A very important step towards standardization and harmonization has been the development by the Adenovirus Reference Material Working Group (ARMWG) of an adenovirus standard [209] that is widely used by the industry. The Adenovirus Reference Material (ARM) is currently distributed through the ATCC.

Typically, small-scale virus cultivation processes implemented in disposable culture-ware are monitored with off-line sampling. For these processes, process parameters such as pH are not typically controlled, and process definition is required to ensure that the pH remains within a critical range during the

cultivation process. As the production scale increases, so does the need for a tighter process control. The cultivation of virus can be controlled by on-line monitoring methods to assure batch-to-batch reproducibility. Cell concentration at infection is a critical process parameter to ensure maximum and reproducible volumetric virus production. Although off-line methods can be used to monitor cell concentration, an on-line method to trigger the infection point is very useful for large-scale stirred tank bioreactor processes. The status of infection has been monitored using a variety of non-invasive on-line measurements to provide useful information. Particularly, the oxygen uptake rate (OUR) and the capacitance signal have been correlated to viable cell growth prior to infection [181, 210]. At infection both signals are affected. An increase in respiration has been noted together with an increase in capacitance measurements [177, 181, 211]. This correlates well with an increase in cell metabolic activity and an increase in cell size upon infection. In an attempt to measure the kinetics of infection of a GFP-expressing AdV, a fluorescent probe has been designed by Gilbert et al [212]. This probe was used to monitor the AdV production kinetics and as a tool to identify the appropriate harvest time. At-line monitoring of GFP cells by flow cytometry has given valuable information on AdV production kinetics [213]. These monitoring tools have however been of limited success in providing indications on the relative efficiency of AdV productions.

5.2. Purification

The development of fast and efficient purification procedures that permit high recovery of infectious particles, removal of host cell proteins and DNA, clearance of unpackaged viral DNA and also concentration of virus formulations is essential for their use in vaccination and gene therapy. As impurities and contaminants can induce immunological and biological responses, they must be removed to comply with strict regulatory guidelines. The regulations for human bioproducts are much more rigorous than for animals. These increases the complexity of downstream processes, including polishing steps to make sure the viral product do not contain any detrimental element for human health (**Figure 1. 9**).

Figure 1. 9: Virus purification steps.

Viruses are quite large when compared with biomolecules such as proteins, peptides, glycoproteins, sugars and nucleic acids that are inevitably present in lysates harvested from virus-infected cells. In fact, viruses really should be considered molecular assemblies. While proteins typically range from 0.005 to $0.08x10^6$ Da, viruses generally exceed $5x10^6$ Da and can have a size of up to 1000 nm. Purification strategies based on viral size have included a variety of methods such as density-gradient ultracentrifugation, ultrafiltration, precipitation, two-phase extraction systems and size exclusion chromatography (SEC) [102, 214, 215]. High-speed preparative centrifugation (ultracentrifugation), using cesium chloride (CsCl) or sucrose gradients, has traditionally been used to process many viral vector preparations.

Virus purification protocols that are more amenable to scale-up generally involve some type of chromatographic procedure. Adsorption of virus particles to solid phases, in fact, provides a convenient and practical choice for fractionating and recovering viruses from cell and media contaminants. While they are somewhat imperfect in their selectivity, adsorption methods do offer several important advantages in large-scale virus purification: (1) high flow rates can be used, thus limiting the processing time, (2) biological activity of labile viruses is often preserved, since mild conditions are generally used to elute the virus from the chromatographic matrix, (3) scale-up is relatively easy, (4) large volumes of cell lysates can be processed and (5) the cost of operation is relatively low. However, it is important to remember that the design of suitable selective chromatographic protocols for virus purification must take into account the structure and physical and chemical surface properties of the viruses [102, 214, 215].

Purification is not within the scope of this thesis. For a more detailed assessment of the virus purification processes described in the literature it is recommend the reading of published reviews [102, 214, 215].

5.3. Formulation and storage

All vaccines are susceptible to damage by elevated temperatures and many are also damaged by freezing. The distribution, storage, and use of vaccines therefore present challenges that could be reduced by enhanced thermostability, with resulting improvements in vaccine effectiveness.

Lyophilization has been the most successful approach used for the development of stable live virus formulations [216]. But when compared to refrigerated liquids they are at a clear disadvantage in terms of cost, complexity and ease of administration (**Table 1.12**). Despite the drawbacks of lyophilization it is usually required for long-term storage of live viruses. Frozen storage of liquid formulations is one alternative to lyophilization; however, this approach significantly increases the difficulty and cost of maintaining an effective cold chain during distribution.

Table 1.12: Advantages and disadvantages of Liquid and Lyophilized formulations.

Formulation	Advantages	Disadvantages
Liquid	- Convenient - Cost of goods (economical) - Competitive - Applicable to many commercially available delivery devices	- Less stable than lyophilized formulations
Lyophilized	- More stable than liquid formulations - Can achieve higher concentration formulation	- Cost of goods (expensive) - More complex process and technical transfer - Limited commercial contract capacity

(Source: [217])

Historically, viral formulations have consisted of glycerol (10-50% v/v) in phosphate or Tris buffers with additions of other excipients such as salts, sugars and bovine serum albumin. It has been shown that the addition of cryoprotectants to viral suspensions (e.g. glucose, sucrose, sorbitol, and trehalose) decreases the loss of infectivity of viral vectors and enhances their

stability during freeze-drying and storage [217-219]; furthermore, pH is a critical factor for the maintenance of viral titers [220, 221].

To maintain the viral characteristics several formulations have been developed that allow the long term storage in liquid and lyophilized form [217, 218, 222] from several months to more than one year in refrigerated conditions (2-8ºC). For more information on this topic we recommend the reading of the following references [223-226].

Besides the stability during viral vaccines storage, the process of production can also affect the final virus potency, as the stability of enveloped viruses can be compromised in cell culture bulks due to temperature. Previous studies from our laboratory have shown significant increase in the intrinsic retrovirus stability, when production was carried out at high concentrations of glucose or fructose [12]. It was also observed that, independently of the osmotic agent used, higher medium osmolalities enhanced retrovirus stability [13]. Ideally, stabilization approaches should be incorporated into vaccines early in the development process.

Significant changes in the stability profile may occur following exposure to temperature stresses exerted through fluctuations in handling or storage conditions. The temperature sensitivity of vaccines has led to the development of cold chain requirements for all vaccines [227]. There was a great deal of interest in making vaccines heat stable and removing them from cold chain storage to facilitate outreach and to help manage the impending impact of the introduction of new vaccines in countries with limited cold chain capacity. There was also a growing body of evidence that cold storage facilities and transportation methods in both developing and industrialized countries often inadvertently expose vaccines to freezing temperatures [228].

Because of this, the stability characteristics of each vaccine must be determined empirically through testing, and the storage and handling conditions be defined to ensure that, over the stated shelf-life of the vaccine, minimum standards of potency, identity, and purity continue to be met [227].

6. Scope of the thesis

The work presented in this thesis focused on the development and improvement of new platforms for production of valuable virus products with impact on human and animal health.

Different cell lines were assessed for the production of Adenovirus vectors (AdV) and Peste des Petits Ruminants virus (PPR) and the culture parameters affecting viral production were determined for each system. Moreover, since viral stability during shipping and storage is one of the main concerns for vaccine effectiveness, different approaches were also evaluated and applied to improve PPR vaccine stability.

A schematic representation of the main aims proposed for this thesis, as well as the strategies that were employed to address them, are summarized on **Figure 1.10**.

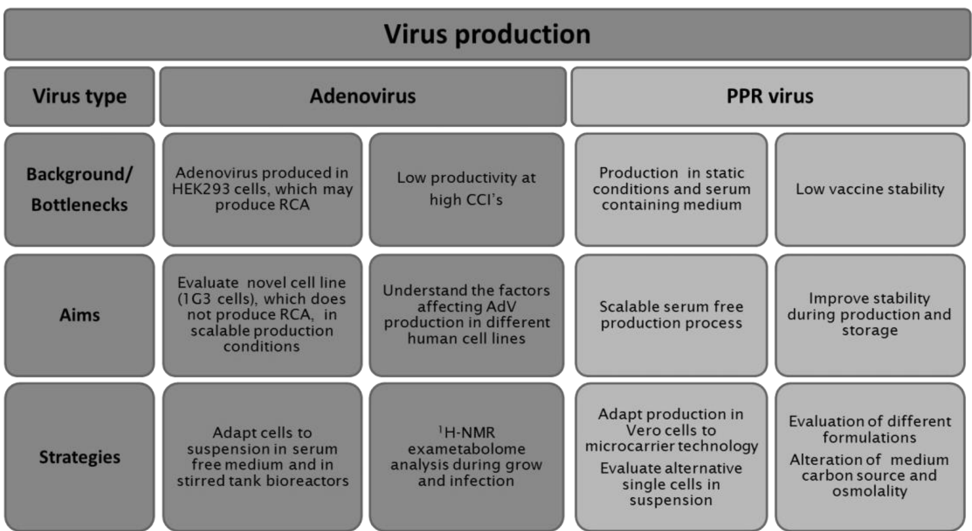

Figure 1.10: Diagram of the main aims and strategies followed in this this thesis.

The strategies followed attempted to meet the new tendency of acceptance benchmarks for an ideal production process: i) use of safe cell lines; ii) use of animal component-free culture medium and iii) rapid production process that yields high concentrations of the product with great quality in a cost effective way. We believe that our findings provide invaluable knowledge for rational viral vaccine bioprocess development. The processes developed herein can be applied to other producing systems by filling the gaps in the production process of human and animal biotherapeutics.

7. Acknowledgments

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8. Author contribution

Ana Carina Silva wrote this chapter based on the referred bibliography and by adapting the above mentioned publications.

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Online Databases:

World Organisation for Animal Health
http://www.oie.int/
Journal of Gene Medicine Clinical Trial Database
http://www.abedia.com/wiley/index.html
The Center for Food Security and Public Health
http://www.cfsph.iastate.edu
BCC Research
http://www.bccresearch.com/
MSD Animal Health
http://www.merck-animal-health.com
Swiss Institute of Bioinformatics, ViralZone[1]
www.expasy.org/viralzone
National Institutes of Health, ClinicalTrials.gov
http://clinicaltrials.gov

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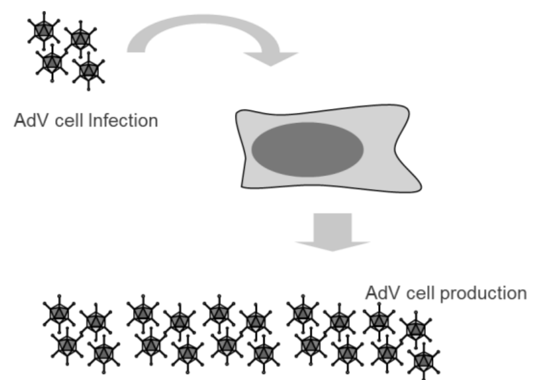
Chapter 2

Human Amniocyte-derived Cells for the Production of Adenovirus Vectors



This part was based on the following manuscript:

Human amniocyte-derived cells are a promising cell host for adenoviral vector production under serum free conditions, Silva, A.C.*, Simão, D.*, Küppers, C.*, Lucas, T.*, Sousa, M.F.Q., Cruz, P.E, Kochanek, S., Carrondo, M.J.T. and Alves, P.M., (2015) *Biotechnology Journal,* 10(5): 760-71 (*authors contributed equally).

Abstract

Recombinant adenovirus vectors (AdV) have been used for the development of vaccines, as gene therapy vectors and for protein production. Currently, the production of clinical grade batches of recombinant E1-deleted Adenovirus type 5 vectors (HAdV-5) is performed using human derived HEK293 or PER.C6 cell lines. In this work we describe the generation of new human amniocyte-derived cell line named 1G3 and show that it can be used as a very promising cell host for AdV production in serum-free conditions, allowing for production in high cell concentration cultures and avoiding the typical cell density effect observed for HEK293. By design this cell line makes the generation of replication competent adenovirus (RCA) during production of E1-deleted Ad vectors very unlikely. The impact of the culture system (static versus agitated) and AdV infection parameters such as multiplicity of infection (MOI), time of harvesting (TOH) and cell concentration at infection (CCI) were evaluated and compared with HEK293. Using stirred tanks bioreactors, it was possible to grow 1G3 cells to cell densities of up to 9 x 10^6 cell/mL using serum-free media. Moreover, without a medium exchange step at infection, a 3-fold increase in AdV volumetric titers was obtained, as no cell density effect was observed at CCI 3. Overall our results clearly demonstrate the potential of the human amniocyte-derived newly established cell line 1G3 for AdV production in a serum-free scalable process, paving the way for further process improvements based on fed-batch or perfusion strategies.

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1. Introduction

Remarkable progress has been made during the last two decades in the development and application of adenoviral vectors (AdV) for gene therapy and vaccination. In many cases, AdV became the vector of choice due to their ability to replicate at high titers in complementary cell lines and to effectively deliver therapies such as vaccines, cancer therapeutics, and therapeutic genes [1-3], establishing itself as the most widely used delivery vector in gene therapy clinical trials [www.wiley.com/legacy/wileychi/genmed/clinical/].

Currently, the clinical grade recombinant E1-deleted human adenovirus type 5 (HAdV-5) vectors for gene therapy and vaccination purposes are produced in cell lines based on human embryonic kidney cells following immortalization with sheared HAdV-5 genomic DNA and containing the first 4344 nucleotides of HAdV-5 including the E1 functions (HEK293 cells) [4] or on human embryonic retinoblasts transformed with HAdV-5 E1A and E1B encoding sequences (PER.C6 cells) [5]. Safety concerns have been described for HEK293 cells especially regarding their tumorigenicity and their tendency to generate replication competent adenovirus (RCA) at larger scales of production [6, 7]. Poor documentation concerning their isolation and banking also raises special concerns from the regulatory authorities. The PER.C6 cell line has been widely adopted by the industry for AdV production, mainly due to its improved documentation of history and properties [5]. However, in two publications regarding PER.C6 cells, the generation of unusual vector recombinants have been observed, resulting in vector specimens carrying and expressing E1 functions [8, 9]. Moreover, PER.C6 prices and licensing are also prohibitive for academic institutions, limiting the progress of R&D for new clinical gene therapy and vaccines application using these cells.

More recently, human amniocytes were identified as an alternative cell source for the generation of stable cell lines following transformation with E1 [10] and E1/pIX [11] functions. The design of the E1 DNA construct in one of the cell lines (N52.E6) was similar to that of PER.C6 cells [5], in principle excluding the generation of RCA due to the absence of any overlap sequence between vector DNA and the integrated E1 region. Several other cell lines were established under the rational of reducing the homology of their viral DNA

sequences with viral vectors genome (reviewed in [12]). This strategies have been applied using different human parental cell types namely lung carcinoma (A549 cells) [13, 14], embryonic lung [15], embryonic retinoblasts [5, 16], and HeLa cells [17, 18]. Unfortunately, the HeLa cell line is not allowed for commercial use because of its high tumorigenicity.

Since Garnier et al. [19] used HEK293 cells, several efforts have been made worldwide towards establishing scalable processes suitable for commercial AdV manufacturing. Several variables and parameters have been shown to affect AdV production, such as temperature, pH, cell concentration at infection (CCI), multiplicity of infection (MOI), time of harvesting (TOH), culture medium (serum containing versus serum-free media), cell culture system (adherent, aggregated and suspension cells from small culture flasks to high volume bioreactors) [20, 21]. A significant amount of literature exists trying to cope with the cell density effect observed for infections at CCI higher than 1 $x10^6$ cell/mL, a phenomenon that leads to a significant decrease in cell specific virus yields [20, 22-25]. Approaches to maintain AdV cell specific titers at increased cell densities consist in infecting the cells after medium exchange [23, 26, 27] or under perfusion [28-30]. However, these procedures add the extra complexity of a cell separation step and increase the production costs due to medium exchange. Other approaches consist of the use of a fed-batch strategy, using non-ammoniagenic medium, or cell cycle synchronization among others, but its success has been limited [1, 2, 22-24, 31-35]. In this way, the cell density effect issue still needs to be further addressed for HEK293 and PER.C6 cells and AdV production.

For manufacturing purposes, especially for large-scale production, suspension adapted cell lines are more convenient, improving the scalability of the process. Furthermore, suspension cell lines can be cultured in serum-free media and are devoid of any bovine-derived additives facilitating downstream purification, as well as certification of clinical-grade batches.

The main objective of this work was to assess the performance of the newly developed human amniocyte-derived 1G3 cell line for the production of HAdV-5 and to evaluate the possibility for development of a scalable and serum-free bioprocess for HAdV-5 production. Cell cultivation and HAdV-5 production was evaluated in different culture systems: T-flask, shake flask and controlled stirred tank bioreactor (STB). The impact of MOI, CCI and TOH on HAdV-5

productivity was studied and directly compared to HEK293 cells. The results demonstrate that the 1G3 cell line can be a promising candidate for scalable production of HAdV-5 in serum-free medium, using a batch bioprocess mode and infecting at higher CCIs, paving the way for further improvements in volumetric productivities based on fed-batch or perfusion modes as shown for several virus systems namely baculovirus [36-38], influenza [39] and even in other studies with AdV [28, 29] .

2. Materials and methods

2.1. Generation of pSTK146UBE2I, used for immortalization of primary human amniocytes

Plasmid pBKSII E1B, containing the HAdV-5 E1B promoter and E1B coding sequence and SV40 sequences (intron and 3´ UTR), was modified by site-directed mutagenesis to remove the splice donor (SD) at nt. 3510 of HAdV-5 and to introduce a NdeI restriction site. The resulting plasmid, named pBKSII E1B QC NdeI, was digested with BamHI and NdeI to delete a 1 kb fragment containing the SV40 DNA sequences. The human UBE2I intron was isolated by PCR using as template genomic DNA isolated from low passage human N52.E6 cells [10] and using oligonucleotides #73 (5´-gttcagCATATGcaggtacggggcctccgcctctg-3´) and #74 (5´-TCAAGGTGGGGGAGGGTtctgtgccagagacaaaaacacaagac-3´). The resulting PCR product, named "PCR intron", was flanked by a NdeI site (underlined in oligo #73) and codon-optimized Ad5 sequences (nt. 3595 to nt. 3612) potentially encoding the C-terminal part of E1B 84R [40] at the 5´or 3´end (capital letters in oligo #74), respectively. The mutagenesis would also result in an amino acid exchange by replacing amino acid 5 from the end (Gln) with an Asn and in the introduction of a stop codon.

The 3´ UTR of UBE2I was isolated using oligonucleotides #75 (5´-gaACCCTCCTCCACCTTGAATTGCCCGTTTCCATACAGGGTC-3´) and #76 (5`-ctggatccGCGGTGGGGCTGCAGGTG-3´(SEQ ID NO: 29)) resulting in a PCR product named "PCR 3´ UTR", which is flanked by the same Ad5 sequences (nt. 3595 to nt. 3612) as mentioned above and a BamHI restriction site (underlined in oligo #76) at the 5´ or 3´ end, respectively. The overlapping Ad5 sequences at the 3´end of "PCR intron" and at the 5´end of "PCR 3´ UTR" allowed fusion of these two PCR products using oligonucleotides #73 and #76. The resulting

PCR fusion fragment flanked by NdeI and BamHI was then inserted into the NdeI and BamHI sites of pBSK E1B QC NdeI resulting in plasmid pBSK E1B UBE2I.

To generate pSTK146 UBE2I, the BglII/ BamHI fragment from pBSK E1B UBE2I containing the UBE2I intron, the C-terminal part of E1B 84R and UBE2I 3´ UTR was subcloned into the BglII and BamHI sites of pSTK146. The resulting plasmid was named pSTK146UBE2I. A simplified map is shown in (Figure 1A).

2.2. Cell lines and culture media

The HEK293 cell line was purchased from ATCC (CRL-1573). The generation of the new 1G3 cell line was performed essentially as in [10] and is described in more detail in the results section. HEK293 and 1G3 cells were maintained in DMEM with 10% FBS (v/v) and 4 mM of glutamine (all from Invitrogen, UK). Cells were sub-cultured twice a week in T-flasks and maintained in a humidified atmosphere at 37°C with 5% CO_2 in air. For cell harvesting, the monolayer was washed with phosphate-buffered saline followed by incubation with 0.05% trypsin-EDTA (Invitrogen, UK) until cell detachment became evident.

2.3. Stirred Cultures

Cells were adapted to grow in suspension cultures as single cells in serum-free Ex-Cell™ 293 medium (SAFC Biosciences, UK) supplemented with 4 mM glutamine (Invitrogen, UK). This was performed by successive passing of cells directly in the serum-free medium, starting with adherent cells in mid-exponential phase.

Shake flasks cultures were performed by inoculating 500 mL shake flasks (80 mL of working volume) with 0.5×10^6 cell/mL that were maintained in an orbital shaker at 130 rpm in humidified atmosphere at 37°C with 8% CO_2 in air. pH, dissolved oxygen (DO) and temperature were monitored using the SFR Shake Flask Reader (PreSens GmbH, Germany).

Stirred-tank bioreactor (STB) cultures were performed using Biostat DCU-I Bioreactors with 1 or 2 liter vessels (Sartorius Stedium, Biotech GmbH, Germany) equipped with rushton impellers, which were inoculated at 0.5×10^6 cell/mL and the working volume was set to half of the vessel volume. pO_2 was controlled at 50% air saturation with constant airflow of 0.01 L/min by sequentially varying the nitrogen and oxygen partial pressure in the gas inlet and the agitation rate.

The pH was controlled at 7.2 by aeration with CO_2 gas-mixture and 1 M $NaHCO_3$ at 37°C.

2.4. Cell concentration determination

Cells were counted using a Fuchs–Rosenthal haemocytometer chamber with trypan blue exclusion method.

2.5. AdV production

The vector used consisted of an E1-deleted HAdV-5, expressing green fluorescent protein as transgene (HAdV5-GFP). This vector is based on plasmid pGS66 that contains HAdV-5 sequences from nt 1 to 440 and from nt 3523 to 35935 [10]. In this plasmid the E1 region is replaced by an expression cassette consisting of the hCMV immediate early promoter, a cDNA coding for the eGFP protein and the SV40 polyA signal. HAdV5-GFP vector was rescued by cleavage of the plasmid DNA with SwaI (flanking the adenovirus ITRs) followed by transfection in N52.E6 cells [10], and by production and purification with CsCl gradients according to standard procedures.

For static cultures, cells were inoculated at 6 x 10^4 cell/cm^2 in DMEM supplemented with 10% FBS (v/v) and 4 mM of glutamine and allowed to grow for 3 days. At a confluency of 70-80%, infection was performed with a MOI of 5 infectious particles (ip) per cell by exchanging the culture media with fresh media containing the vectors.

In stirred cultures, for evaluation of the impact of MOI and TOH in 1G3-mediated AdV production, cells were grown to a concentration of 1 x10^6 cell/mL and infected with MOIs of 0.05, 0.5, 5, 10 and 20. To evaluate the effect of CCI, 1G3 and HEK293 were infected at 1, 2, 3 or 5 x10^6 cell/mL with a MOI of 5.

Extracellular vector particles were collected from the cell supernatant after centrifugation at 1000 g for 10 min at 4°C. For intracellular vector harvesting, the resulting cell pellet was resuspended in lysis buffer (Tris/HCl 10mM, pH 8.0 and 0.1% (v/v) Triton-X 100) disrupting the cells and clarified at 3,000 g for 10 min at 4°C. All samples were stored at -85°C.

2.6. AdV titration

AdV titration was performed by monitoring the expression of GFP reporter gene after infection of HEK293 cells seeded in 24-well plates as previously described [24]. Briefly, cells were seeded at 0.25 x 10^6 cell/well and infected 24 hours post infection (hpi) with 1 mL of serial dilutions of the AdV samples in medium (DMEM with 10% (v/v) FBS). Cells were trypsin-detached at 17-20 hpi and the percentage of GFP-positive cells determined by flow cytometry (CyFlow Space, Partec, Germany).

2.7. Assessment of the generation of replication competent adenovirus (RCA) during production

The generation of RCA was evaluated by performing serial passages in two cellular clones (1G3 and 1D9 cells) of an E1-deleted adenovirus vector expressing EGFP (HAdV5-GFP) and finally using A549 and HeLa cells as indicator cells for the detection of RCA generated during serial passage. This assay was performed in two different ways, in high and a low infection formats.

In the first format, each cell line was seeded in 6-well dishes at 1.5 x 10^6 cells per well (10 wells per cell line). Cells were infected with HAdV5-GFP at a multiplicity of infection (MOI) of 10 infectious particles per cell and harvested after 48h. The cells were lysed by freezing/thawing three times. 10% (v/v) of each cell lysate was used to infect cells of the same cell line for another infectious cycle. This procedure was repeated for a total of 15 passages.

In a second assay format, cells were infected with 0.1% (v/v) of cell lysate from the previous passage (low infection format) and harvested when a cytopathic effect (CPE) became visible after 5-8 days, thus allowing for several rounds of replication. This procedure was also repeated for 15 passages.

RCA detection was performed essentially as described previously [5]. The assay was performed on human A549 and HeLa cells, which do not allow for replication of E1-deleted Ad vectors. Only in the case of RCA generation, a full infectious cycle can occur, resulting in a CPE. The final lysate after serial passage was used for infection of HeLa cells for 4 days. Afterwards, the HeLa cells were lysed by 3x freezing/thawing and the lysate was added to A549 cells for 10 days. A visible CPE on A549 would indicate the presence of RCA, as an E1-deleted Ad vector would not replicate in either HeLa or A549 cells. To test

the detection limit of this assay, control HeLa dishes were infected with lysates spiked with HAdV-5 wild-type particles at very low multiplicity of infection. The assay has been found sensitive enough to detect 6 wild-type particles per infected HeLa dish.

After 15 vector passages, no RCA was detected in any lysate of amniocyte-based cell lines (a total of 40 lysates were tested), while three in 20 final lysates of 293 cells were found to contain RCA.

2.8. Cell growth characterization

The maximum specific growth rates (μ_{max}) were determined using the equation: $\mu_{max}=[\ln(X_v/X_{v0})]/t$, where t is the culture time and X_{v0} and X_v are respectively the viable cell concentration at the initial time and for time t. Doubling time (td) was calculated using the equation $td=[\ln(2) / \mu_{max}]$.

2.9. Metabolic characterization

Glucose (Glc), Lactate (Lac) and Glutamine (Gln) were quantified with an automated enzymatic assay (YSI 7100 Multiparameter Bioanalytical System, USA). Ammonia ($NH4^+$) was quantified by enzymatic assay K-AMIAR (Megazyme, Ireland). The specific metabolic rates (qMet) were calculated using the equation: $qMet= \Delta Met/(\Delta t.\Delta X_v)$, where ΔMet is the variation in the metabolite concentration during the time interval Δt and ΔX_v is the difference in viable cell concentration during the same time interval. The apparent metabolic yield (YMet1/Met2) was calculated as the ratio between q_{Met1}/q_{Met2}. The oxygen uptake rate (OUR) was determined using the equation: OUR=KLa.(C*-C), where the KLa (determined using the gassing-out method [41]) is the oxygen transfer coefficient, C* and C are the oxygen concentration at saturation and in solution, respectively. The oxygen specific consumption rate (qO_2) was calculated using the equation: $qO_2=OUR/X_v$.

3. Results

3.1. Rationale and generation of pSTK146UBE2I

Previously we had generated the N52.E6 cell line by immortalization of primary human amniocytes with plasmid pSTK146 [10]. This plasmid, containing the E1 region of HAdV-5 from nt 505 to 3522, expresses the E1A functions from the murine PGK promoter and the E1B 55K and 19K proteins from the endogenous E1B promoter. To prevent generation of RCA during production of E1-deleted Ad vectors in E1 complementing producer cells, most of the E1B intron, the E1B splice acceptor (SA) and the 3' UTR (overlapping with the protein IX gene) had been replaced with the corresponding elements from SV40, i.e. intron, SA and polyA signal [10]. Following transient transfection of pSTK146 in HeLa cells we now noted in Western blot analysis using an antibody for detection that recognizes the N-terminus of E1B that relatively little 55K E1B protein was produced, while a prominent band was visible at about 37 kDa, while in N52.E6 cells only a 55 kDa (full-length) E1B signal was visible (data not shown). RT-PCR analysis indicated that the E1B truncation was due to an aberrant splicing event involving a splice donor (SD) downstream from the known E1B SD (SD1) and the SV40 SA (data not shown). With the idea to potentially increase immortalization efficiency (by expressing larger amounts of full-length E1B 55K protein) we replaced the SV40 intron/SA/polyA elements with a short intron, including SD and SA, and a 3'UTR of the human UBE2I gene. In addition, we inserted a short sequence from the 5'UTR of the pIX gene, potentially allowing for expression of the minor E1B 84R protein [40]. This sequence was codon-optimized, which as a side effect considerably reduced homology with the natural pIX sequence (**Figure 2. 1**). As a consequence, in a stretch of 21 nucleotides, only 15 nucleotides were homologous to the HAdV-5 sequence and with not more than 3 nucleotides in a row, making recombination by homologous recombination extremely unlikely.

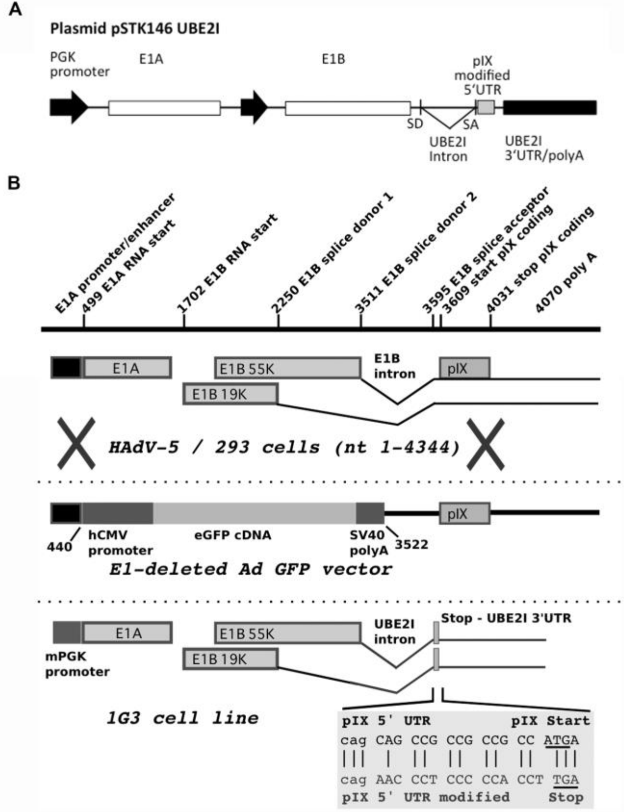


Figure 2. 1: Rationale for 1G3 cell line generation. **A.** Simplified map of pSTK146UBE2I that was used for immortalization of primary human amniocytes. The E1A region is under control of the murine PGK promoter. The E1B region is under control of the endogenous E1B promoter. Splice donor (SD) and splice acceptor (SA) of the UBE2I intron are indicated. **B.** Scheme of the E1 region in HAdV-5 wildtype virus and in HEK293 cells, of an E1-deleted Ad vector expressing eGFP and of the new 1G3 cell line described in this manuscript. The top panel indicates relevant features of HAdV-5 (NCBI Reference Sequence AC_000008.1).
The region of significant overlap between the genome of the E1-deleted AdV5-GFP vector and left part of the HAdV-5 genome in HEK293 cells potentially resulting in homologous recombination and the generation of RCA is indicated with red crosses. The numbers 440 and 3522 delineate the deletion of the E1 region in typical E1-deleted Ad vectors, and in particular in the eGFP -expressing vector AdV5-GFP used in this study.
The insert at the bottom of the figures provides the details of the modified 5' UTR of the pIX gene present in the 1G3 cell line. The small letters indicate the intron and the capital letters the exon sequence (i.e. indicating the location of the splice acceptor). The ATG start codon of the pIX coding sequence is indicated, which is modified in the 1G3 cell so that a TGA stop codon is introduced instead.

3.2. Generation of cell line 1G3

The generation of permanent amniocyte cell lines was approved by the ethics committee of the University of Ulm. Following written informed consent of the donors, primary cells obtained as a side product from diagnostic amniocentesis were cultivated, basically as described in [10]. Further details of

the cultivation of primary cells are provided in [42]. The primary cells had a normal karyotype and tested negative for HIV, HBV, HCV, CMV and EBV.

Primary cells, cultivated following standard procedures, were transfected with plasmid pSTK146UBE2I following its linearization with BspHI, which cleaves in the plasmid backbone, and using linear polyethyleneimine (PEI) as transfection reagent. Primary cells in 6 cm culture dishes and at a confluency at 50-70% were transfected with 2µg linearized plasmid DNA per dish in 150mM NaCl using linear polyethyleneimine (PEI), pH 7, at a N/P (nitrogen/phosphorous) ratio of 45 for transfection. Expression of the plasmid-encoded HAdV-5 E1 genes after chromosomal DNA integration is sufficient to achieve immortalization of the cells. Therefore, additional selection was not required. Twenty-four hours after transfection cells were transferred to 14 cm dishes using TrypLE Select (Gibco) for detachment. Several weeks after transfection, when foci of immortalized cells became visible, individual cell clones were isolated and expanded. These were subsequently tested for complementation of an E1-deleted Ad vector. Clones with good productivities were single-cell cloned by limited dilution. From these the 1D9 and the 1G3 cell lines were selected for further analysis. Further details of the procedure are described in [42]. The 1G3 cell lines was adapted to growth in suspension culture as described in the Material & Methods section and further analysed in the present study.

3.3. Human amniocyte 1G3 cell line produces significant amounts of AdV in static culture conditions without RCA formation

To determine the potential of the 1G3 cell line for AdV production, its specific cell growth and metabolic properties were initially studied in a static culture system and in comparison with the widely studied HEK293 cell line.

The obtained cell growth profiles showed that both cell lines had similar growth rates (μ_{max}), although 1G3 cells were able to attain a higher maximum viable cell concentration (Xv_{max} of $8x10^5$ cell/cm^2) (**Table 2. 1**). Concerning metabolic activity, 1G3 cells presented lower glucose uptake rates (q_{Glc}) resulting in smaller lactate production rates (q_{Lac}) when compared to HEK293 cells. However, there were no differences observed for the molar ratio $Y_{Lac/Glc}$ between the two cell lines, suggesting similar metabolic profiles in terms of

glycolysis and oxidative metabolism. The molar ratio $Y_{Glc/Gln}$ was lower for 1G3 cells (3.2 and 4.9, respectively for 1G3 and HEK293), indicating that 1G3 cells presented an increased glutamine metabolism over glucose as carbon source than HEK293 cells in this culture system and serum containing conditions (**Table 2. 1**).

Table 2. 1: Cell growth characterization for the two cell lines in T-flask, shake flask and STB.

Parameters	T-Flask		Shake Flask		STB	
	1G3	HEK293	1G3	HEK293	1G3	HEK293
Xv_{max} (^{a}x10^5 cell/cm^2; ^{b}x10^6 cell/mL)	8.2 ± 0.8^a	7.0 ± 0.7^a	7.7 ± 0.8^b	5.4 ± 0.6^b	9.2 ± 0.9^b	6.1 ± 0.5^b
μ_{max} (h^{-1})	0.029 ± 0.001	0.030 ± 0.001	0.024 ± 0.002	0.022 ± 0.001	0.025 ± 0.003	0.025 ± 0.004
t_d (h)	23.8 ± 0.5	23.0 ± 0.4	28.8 ± 2.2	32.1 ± 2.0	27.6 ± 1.2	27.6 ± 1.4
q_{Glc} (10^{-11} mmol/Cell.h)	14.0 ± 1.0	24.5 ± 1.1	9.9 ± 1.3	8.0 ± 0.3	11.7 ± 0.3	16.1± 0.5
q_{Lac} (10^{-11} mmol/Cell.h)	23.7 ± 2.6	38.7 ± 3.3	12.1 ± 3.1	10.8 ± 1.9	7.2 ± 0.4	12.4 ± 0.3
q_{Gln} (10^{-11} mmol/Cell.h)	4.3 ± 0.5	5.0 ± 0.6	1.5 ± 0.1	1.9 ± 0.4	1.8 ± 0.2	2.2 ± 0.4
$Y_{Lac/Glc}$ (mol/mol)	1.7 ± 0.2	1.6 ± 0.2	1.2 ± 0.4	1.4 ± 0.4	0.7 ± 0.1	0.8 ± 0.1
$Y_{Glc/Gln}$ (mol/mol)	3.2 ± 0.4	4.9 ± 0.6	6.5 ± 1.0	4.2 ± 0.9	6.5 ± 0.9	5.6 ± 1.5

Xv_{max} – maximal viable cell concentration (the error corresponds to the 10 % error associated to cell counting method); μ_{max} – specific growth rate and t_d – duplication time;, q_{Glc} – specific glucose uptake; q_{Lac} – specific lactate production; q_{Gln} – specific glutamine uptake ; $Y_{Lac/Glc}$ molar ratio lactate production/glucose uptake ; $Y_{Glc/Gln}$ – molar ratio glucose uptake/glutamine uptake (the errors corresponds to the standard deviation associated with the parameter estimation).

For assessment of AdV productivity the infection was performed by complete medium exchange in order to exclude any nutrient limitation at time of infection. The maximal AdV productivity was observed at 48 hpi for both cell lines. The obtained results showed that although 1G3 cells enabled efficient AdV replication, producing significant titers (1.0x10^9 ip/mL), the cell-specific productivity of infectious vector particles in this culture system was 2.4-fold lower than for HEK293 (1.7 x10^3 and 4.1x10^3 ip/cell, respectively) (**Figure 2. 2**).

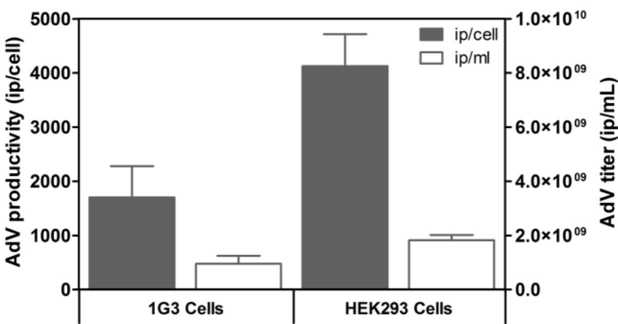


Figure 2. 2: Adenovirus vector (AdV) production in 1G3 and HEK293 cells in static cultures.
AdV productivities (gray) and titers (white) obtained at 48 hpi for 1G3 and HEK293 cells cultured in static conditions. (Mean ± SD, N=2, Error bars represent the standard deviation of quadruplicate sample assay).

The RCA generation was assessed in both cell lines after 15 AdV serial passages and none was detected in any lysate of 1G3 (40 lysates tested), while 3 out of 20 final lysates of the HEK293 cells were found to contain RCA.

3.4. Human amniocyte 1G3 cell line grows in single cell suspension to high concentrations in stirred culture systems

In order to meet the high quantities required for clinical applications, the development of scalable and robust AdV production processes is imperative. In this context, 1G3 and HEK293 cells were adapted to grow in single cell suspension in shake flasks by successive passages in a commercially available serum-free medium (Ex-Cell™ 293). Cell growth and AdV production were evaluated both in shake flasks and STB for 1G3 and HEK293 cells.

For both systems, high viable cell densities were obtained after 120 h in culture (**Table 2. 1**). Although no significant differences were observed for the determined growth rates of the two cell lines, 1G3 cells were able to attain higher maximum cell densities (7.7 and 9.2 x 10^6 cell/mL, respectively, for shake flask and STB) in comparison with HEK293 (5.4 and 6.1 x 10^6 cell/mL, respectively, for shake flask and STB). Under controlled environmental conditions enabled by the STB system a slight increase of the maximum cell densities was observed for the two cell lines relative to the results obtained in shake flask. Also, the obtained $Y_{Lac/Glc}$ ratio was lower for both cell lines in STB cultures relative to shake flask, suggesting an increase of oxidative metabolism, decreasing the reliance on glycolysis as main energy source. The observed $Y_{Glc/Gln}$

ratio was higher for 1G3 cells indicating that these cells, relatively to HEK293, have an increased preference for glucose over glutamine as carbon source (**Table 2. 1**) in suspension cultures and serum-free conditions.

3.5. Human amniocyte 1G3 cell line presented higher AdV productivities than HEK293 cells at CCI 3 in stirred culture systems

In order to maximize AdV production, MOI and TOH were initially optimized in 1G3 cells, by infecting cells at 1 x 10^6 cell/mL in shake flask with different MOIs (0.05, 0.5, 5, 10, 20 and 50) (**Figure 2. 3**). The obtained results identified higher AdV production for MOIs of 5 and 10. Concerning TOH, for these MOIs higher titers were obtained at 48 hpi with similar profiles also observed for HEK293 cells (data not shown).

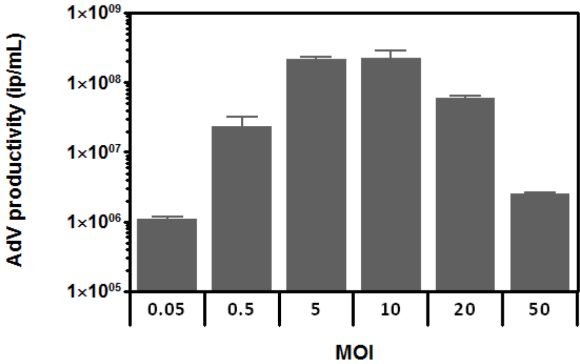


Figure 2. 3: Effect of MOI in AdV titers obtained for 1G3 cells cultured in shake flasks. The cells were infected at different MOIs at a CCI of 1 x 10^6 cell/mL. Data correspond to the maximal titers obtained between 36 and 72 hpi. Error bars represent the standard deviation of quadruplicate sample assay.

AdV production is known to be dependent on the CCI, with lower specific productivities being obtained at higher CCI, which has been described as the "cell density effect" [20] . To evaluate this, 1G3 and HEK293 cells were infected at different CCIs in shake flask (CCI 1, 3 and 5 x10^6 cell/mL for 1G3 cells; CCI 1, 2 and 3 x10^6 cell/mL for HEK293 cells) and STB (CCI 1, 3 and 5 x10^6 cell/mL for both cells). In all cases a MOI of 5 was used, based on the previous MOI optimization.

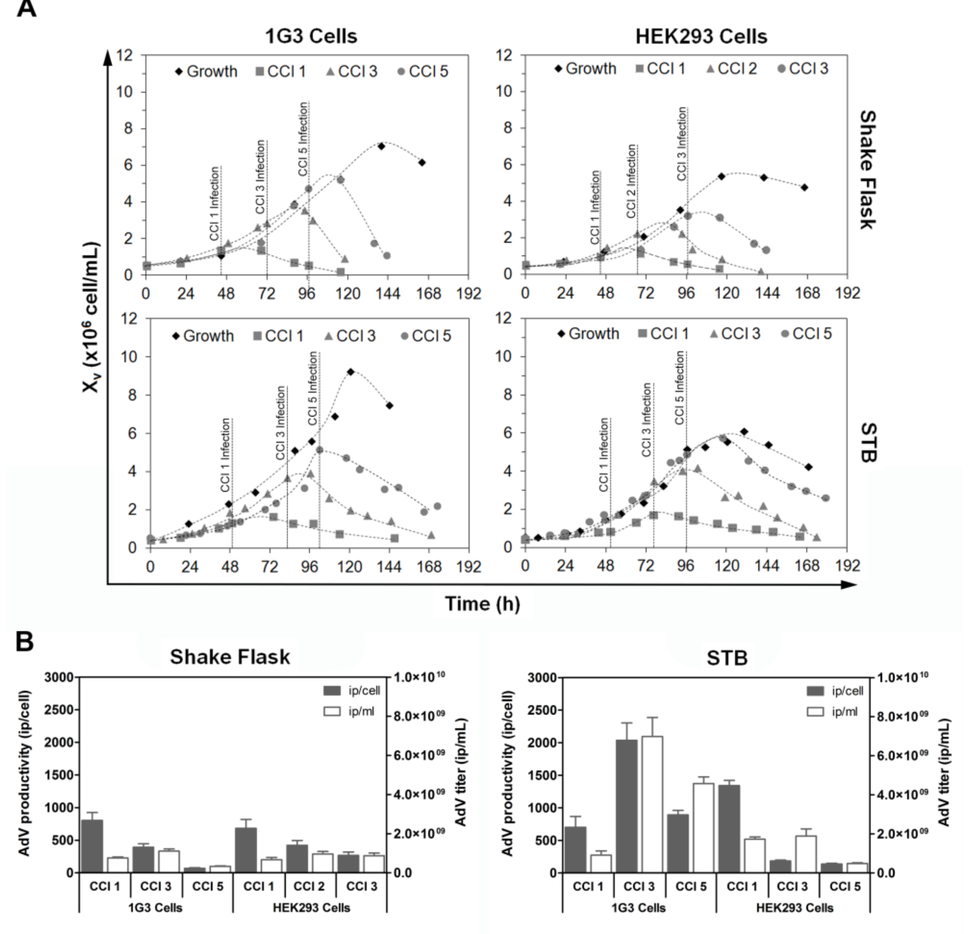
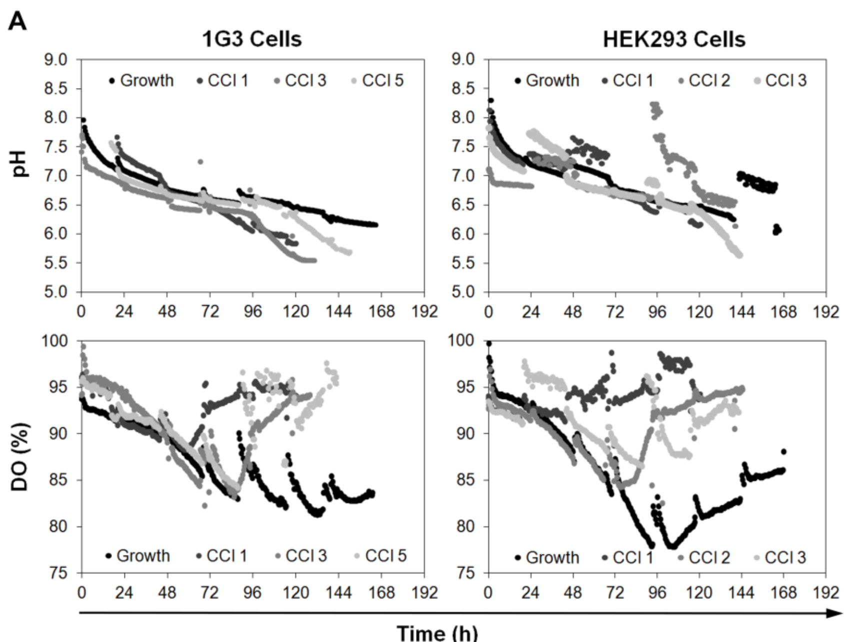


Figure 2. 4: Cell growth and Adenovirus vector (AdV) productivity in 1G3 and HEK293 cells in suspension cultures. **A.** Growth profile of 1G3 and HEK293 cells infected at cell concentration at infection (CCI) of 1, 2, 3 and 5 x 10^6 cell/mL cultured in shake flasks and stirred tank bioreactors (STB). **B.** AdV productivities obtained for 1G3 and HEK293 cells infected at CCI of 1, 3 and 5 x 10^6 cell/mL cultured in shake flasks and STB (Mean ± SD, N=2 for CCI 1 and CCI 3, Error bars represent the standard deviation of quadruplicate sample assay)

Concerning cell growth profiles after AdV infection, only a slight increase in cell concentration was observed during the first 24 hpi for all CCIs, both in shake flask and STB (**Figure 2. 4**), which should be mainly related to the high MOI used in all experiments performed. After this period the cell concentration and viability decreased, which was more pronounced in shake flasks than in STB. In shake flasks, this was also followed by a decrease in pH along culture time, reaching values below 6 (**Figure 2. 5 A**). DO values were always above 75%, indicating no oxygen limitation in the shake flasks experiments during growth or infection for both cell lines. Also, for shake flasks cultures, the specific oxygen consumption, depicted by the qO_2 values determined for both

cell lines, revealed no significant differences during the cell growth phase (**Table 2. 2**). After infection, a decrease in qO_2 was observed, which was even lower for higher CCI.



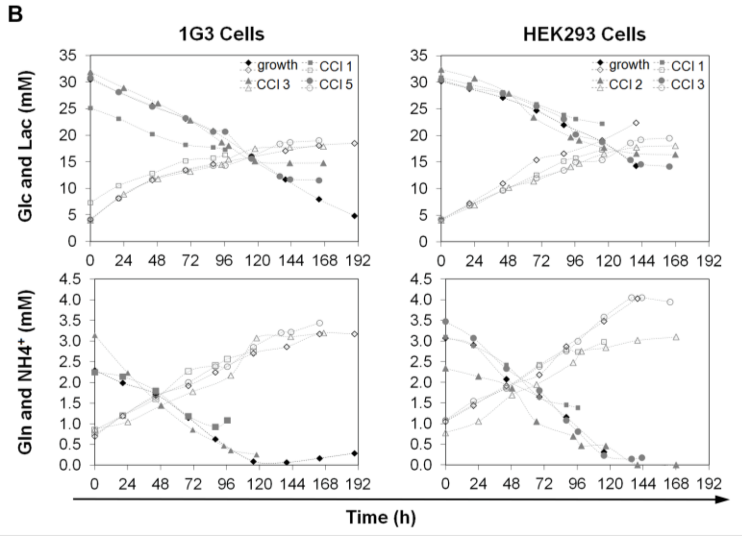


Figure 2. 5: A. pH and DO profiles for 1G3 and HEK293 cells infected at CCI of 1, 2, 3 and 5 x 10^6 cell/mL cultured in shake flasks. **B.** Metabolite profiles for 1G3 and HEK293 cells during growth and infection at different CCIs cultured in shake flasks. Glucose (Glc) and Glutamine (Gln) (full symbols); Lactate (Lac), and Ammonia (NH_4^+) (empty symbols).

Infections performed at CCI 1 in shake flask showed no glucose or glutamine limitations. However, at higher CCIs glutamine was depleted at 48 hpi (**Figure 2. 5 B**), reaching concentrations lower that 1 mM for infections at CCI 3 and vestigial at CCI 5. The determined specific consumption/production rates for all the metabolites analyzed decreased during the infection phase and with the increase in CCIs (**Table 2. 2**), suggesting lower metabolic activity in terms of central energy metabolism pathways. For CCI 3, the molar ratio $Y_{Glc/Gln}$ determined for the infection phase increased, suggesting an increase in glutamine utilization as energy source. This may be explained by the decrease in glucose availability registered at these later stages of culture. Nevertheless, this was followed by a slight increase in the $Y_{NH4+/Gln}$ ratio, indicating lower efficiency in glutamine utilization with higher ammonia production.

Table 2. 2: Metabolic characterization for the two cell lines during growth and infection at CCI'S 1 and 3 in shake flask cultures.

Parameter		1G3			HEK293		
		Growth	CCI 1	CCI 3	Growth	CCI 1	CCI 3
(10^{-11} mmol/Cell.h)	q_{O2}	4.4 ± 0.7	2.3 ± 0.4	1.6 ± 0.2	3.9 ± 0.6	2.8 ± 0.4	1.4 ± 0.2
	q_{Glc}	9.9 ± 1.3	8.8 ± 0.2	6.0 ± 0.4	8.0 ± 0.3	10.3 ± 0.5	4.8 ± 0.6
	q_{Lac}	12.1 ± 3.1	8.8 ± 0.6	5.5 ± 0.2	10.8 ± 1.9	13.1 ± 0.5	5.9 ± 0.6
	q_{Gln}	1.5 ± 0.1	1.3 ± 0.3	0.5 ± 0.1	1.9 ± 0.4	2.1 ± 0.4	0.5 ± 0.2
	q_{NH4+}	1.6 ± 0.4	1.6 ± 0.2	0.9 ± 0.5	1.8 ± 0.2	1.9 ± 0.2	0.9 ± 0.1
$Y_{Lac/Glc}$ (mol/mol)		1.2± 0.4	1.0 ± 0.1	0.9 ± 0.2	1.4 ± 0.4	1.3 ± 0.1	1.2 ± 0.2
$Y_{Glc/Gln}$ (mol/mol)		6.5 ± 1.0	6.6 ± 1.5	12.2 ± 1.5	4.2 ± 0.9	4.9 ± 0.9	9.1 ± 3.0
$Y_{NH4+/Gln}$ (mol/mol)		0.7 ± 0.3	1.2 ± 0.2	1.9 ± 1.1	0.8 ± 0.2	0.9 ± 0.2	1.8 ± 0.6

q_{O2} - specific oxygen uptake, q_{Glc} – specific glucose uptake; q_{Lac} – specific lactate production; q_{Gln} – specific glutamine uptake; q_{NH4+} – specific ammonia production; $Y_{Lac/Glc}$ molar ratio lactate production/glucose uptake; $Y_{NH4+/Gln}$ – molar ratio ammonia production/glutamine uptake.; $Y_{O2/Glc}$ – molar ratio oxygen uptake/glucose uptake; $Y_{O2/Gln}$ – molar ratio oxygen uptake/glutamine uptake (the error corresponds to the standard deviation associated with the parameter estimation); CCI - cell concentration at infection; CCI 1 – 1×10^6 cell/mL; CCI 3 – 3×10^6 cell/mL.

As for AdV production in shake flask, the results showed a significant drop in the specific AdV productivity for CCIs above 1×10^6 cell/mL, for both cell lines (**Figure 2. 4**). Nevertheless, higher specific productivities were obtained

with 1G3 cells at CCI of 3, relatively to HEK293 cells (392 and 267, respectively for 1G3 and HEK293 cells).

The translation of this AdV production bioprocess from shake flask into fully controlled STB resulted in a general increase of the productivities for all tested CCIs (Figure 3). In this system, the same strong negative dependence of specific productivities on CCI occurred for HEK293 with a 10-fold decrease in the specific AdV productivities at the higher CCIs (1342, 182, and 142 ip/cell at CCI 1, CCI 3 and CCI 5, respectively). This effect was completely abolished for 1G3 cells in STB cultures, where in fact an increase of 2.9-fold was observed for CCI 3 relatively to CCI 1 (701 and 2036 ip/cell, respectively for CCI 1 and 3). As for CCI 5, although the specific AdV productivity (892 ip/cell) decreased relatively to CCI 3, it was maintained at similar levels as those obtained for CCI 1. The increase in cell specific AdV productivity in 1G3 cells at CCI 3 resulted in a 3.3-fold increase of the maximum volumetric titers obtained relatively to HEK293.

4. Discussion

In this study, we describe the generation of a new human cell line for production of HAdV-5 based Ad vectors, named 1G3, and we show that this cell line represents a promising and efficient alternative for AdV production to the traditionally used HEK293 or the commercial PER.C6 cell line. By establishing a robust and reproducible bioprocess for AdV production in 1G3 cells, from small scale static culture conditions up to fully controlled and scalable STB, we demonstrated that the attained productivities can be comparable or even higher than in HEK293. Human amniocyte-derived cells have been previously described as efficient platforms for biopharmaceuticals production, namely CAP cells from CEVEC that have shown to be good substrates for recombinant protein production [43] and virus production like influenza [44], with similar productivities to HEK293 cells [45]. However, these cells have only been evaluated for AdV productions in small scale experiments [10]. In order to reduce the chances of RCA generation in AdV production 1G3 cells were transformed with a plasmid containing the Ad5 E1A and E1B genes plus modified sequences from the pIX gene, with little overlap between vector and cellular DNA upon vector production. This resulted in no detectable RCA levels

in a total of 40 independent small scale assays of 15 serial passages of AdV. On the other hand, in HEK293 cells the presence of RCA was detected in 3 out of 20 assays. These results demonstrated that 1G3 cells can offer a safer alternative for AdV production, in comparison with HEK293, due to the restricted viral genome sequences that were inserted in the host cell genome.

In this work, growth and infection studies with 1G3 cells were performed in T-flask, shake flask and STB, in order to demonstrate the potential of 1G3 cells for AdV production. These studies were always performed in parallel experiments performed using HEK293 cells, for a direct comparison of the performance of both cell lines. It was shown that in static conditions 1G3 cells could produce significant titers of AdV ($1.0x10^9$ ip/mL; $1.7\ x10^3$ ip/cell), although slightly lower than HEK293 cells ($1.8x10^9$ ip/mL; $4.1x10^3$ ip/cell). By moving from static towards stirred culture systems, a decrease in the specific AdV productivities for both cell lines was observed, which was more pronounced in HEK293 cells. This occurrence has already been reported for AdV production in HEK293 cells, as well as for the production of other viral vectors in other cell lines [20, 46]. Nevertheless, stirred culture systems are more appropriate for scalable production processes, enabling large-scale production of clinically relevant titers. Adaptation of 1G3 cells cultivation in suspension in a serum-free culture medium was successful, enabling to attain cell densities above 5×10^6 cell/mL within 120 h, which was the maximum cell concentration observed for HEK293 cells and has also been described for PER.C6 cells [27] or CAP cells [39, 44].

Process-related parameters such as MOI, TOH and CCI strongly influence virus productivities and need to be evaluated for each system. The optimal MOI was determined to ensure that the cells were infected with an adequate number of viral particles for maximum volumetric virus production. The highest AdV titers were obtained with MOIs of 5 to 10 and TOH of 48 hpi, similar to those normally applied for HEK293 and PER.C6 cells [20, 21]. Given this, a MOI of 5 was selected as optimal for the CCI studies in order to reduce the amount of AdV stock used for infection, which in the case of industrial processes can help in saving costly certified master virus banks. Extensive literature has described the occurrence of the ‘‘cell-density effect’’ in HEK293 and PER.C6 cultures where the cell specific productivity decreases with an increase of CCI [20, 25, 27]. This effect seems to limit the range of the CCI to $0.5–1x10^6$ cell/mL in order to attain

the maximal productivity. In this work, a decrease of AdV productivity with the increase of CCI was observed for both cell lines in the shake flask culture system. However, in STB this effect did not occur for 1G3 cells, where the specific AdV productivities were similar for CCI 1 and 5 and a 3-fold increase was achieved for CCI 3, resulting in higher titers. Moreover the ratios physical to infectious for 1G3 cells were similar to those obtained for HEK293 cells (data not shown). The experiments for 1G3 cells at CCI 3 and HEK293 cells at CCI 1 were performed twice and the profiles for cell growth and AdV production were similar between independent runs at different cell passages showing the robustness of the cell line for AdV production.

Under controlled conditions 1G3 cells showed the potential to give rise to higher AdV titers. This result shows that 1G3 cells present a different behavior towards the cell density effect than HEK293 cells, a phenomenon which requires further investigation.

For the development of new bioprocesses the Food and Drug Administration (FDA) has created the process analytical technologies (PAT) initiative which encourages designing, analyzing, and controlling of manufacturing processes as early as possible in the development phase [47]. On-line monitoring of essential process parameters such as DO and pH in small devices can prove to be of great value to achieve process and product consistency, as well as process understanding from small scale to bench-scale devices [48, 49] or scale-down [50]. Significant advances have been made equipping small culture devices such as microplates [51, 52], T-flasks [53] and shake flasks [41, 54] with DO and pH sensors. In this work, on-line monitoring of these parameters in shake flask revealed no limitations on oxygen during all cultures. This culture system is widely used in the early development phases for several processes and the more information acquired the better for the design of scale-up strategies. Monitoring DO along culture time in shake flasks enabled to calculate qO_2 for both cell lines, during growth and infection phases, which revealed similar oxygen consumption profiles with a decreased after infection. Moreover, the obtained values are in agreement with those available in the literature for CAP, HEK293 and other cell lines [39, 55]. As for pH, in shake flask it was shown that during infection values lower than 7 were attained, which is below the optimum value described in the literature for AdV production (7.2 for HEK293 [56] and 7.3 for PER.C6 [26]).

In the case of the STB the DO was maintained at 50% air saturation, which is in the range described for most controlled systems reported in the literature for AdV production (30-80% air saturation) [20, 21]. Lower partial pressures of oxygen can contribute to an increased productivity, as it has been reported for Vesicular Stomatitis virus (VSV) production in Vero cells, where high DO conditions led to a decrease in viral productivity due to an increase in reactive oxygen species generation [57]. Thus, the lower levels of DO and a stable pH registered in STB cultures, relative to shake flasks, could be of help to explain the increase in AdV productivities obtained in this culture system.

Additionally, the main carbon sources, glucose and glutamine, were at limiting concentrations at higher CCI's for both cell lines, which could also have affected the AdV productivities, as previously reported for HEK293 cells at CCI 3 [22]. 1G3 cells, like HEK293 and PER.C6 cells exhibited a preference for glucose over glutamine metabolism with a ratio $Y_{Glc/Gln}$ in the order of 5–10 mol/mol [27, 58]. Still, in stirred culture systems the $Y_{Glc/Gln}$ was higher for 1G3 cells than for HEK293 cells, which may suggest that glutamine depletion during infection could have less impact on the AdV productivity of this cell line. In static cultures, the contrary was observed showing that the use of different culture medium with serum and distinctive culture system leads to changes in the cells metabolic behavior. This has been demonstrated in other production systems like avian cells for the production of influenza where changing from roller bottles to bioreactor systems impacts on cell metabolism [46].

It has been reported that feeding with glucose and glutamine could not reconstitute the cell specific productivity at a CCI 3. However, a complete medium exchange at the time of infection could improve the productivity at this cell concentration [22]. Further studies concerning the impact of AdV on the cellular metabolic requirements of both cell lines are on-going using NMR and mass spectrometry. This may provide important cues for metabolic engineering strategies necessary to increase the cell-specific productivity at higher CCI and to understand the differences obtained between 1G3 and HEK293 cells.

Overall, the AdV infection experiments with 1G3 cells showed that (i) infection was most efficient at a MOI of 5; ii) infection at a CCI 3 in STB allowed higher AdV titers; iii) the optimal time of harvest was 48 hpi, corresponding to the higher intracellular AdV concentration. This strategy allowed a 3.3-fold increase in the AdV volumetric production obtained in batch culture in

comparison to HEK293 cells. The results presented showed the great potential of 1G3 cells for AdV production in different culture systems, relevant for the different stages of process development.

5. Conclusions

The development of robust, scalable and safer cell culture systems for viral production is in the focus of both academic and industrial research. In this work we describe the generation of a new human cell line based on primary amniocytes and we show that it is both a good cell host for AdV production and a good alternative to HEK293 cells. The design of the cell line essentially excludes the generation of RCA by homologous recombination. The translation from static cultures to shake flask and then to stirred tank bioreactors demonstrates an easy cell adaptation to different culture systems with maintenance of AdV production yields that allows for a smooth and straightforward scale-up.

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Conflict of interest: Stefan Kochanek licensed the 1G3 cell line to CEVEC Pharmaceuticals, Cologne, after this work had been carried out.

7. Author contribution

Ana Carina Silva together with Daniel Simão participated on the experimental setup and design of cell evaluation, performed part of the experiments, analyzed the data and wrote the chapter. Claudia Küppers and Tanja Lucas performed the work for the development of the cell line used in this work.

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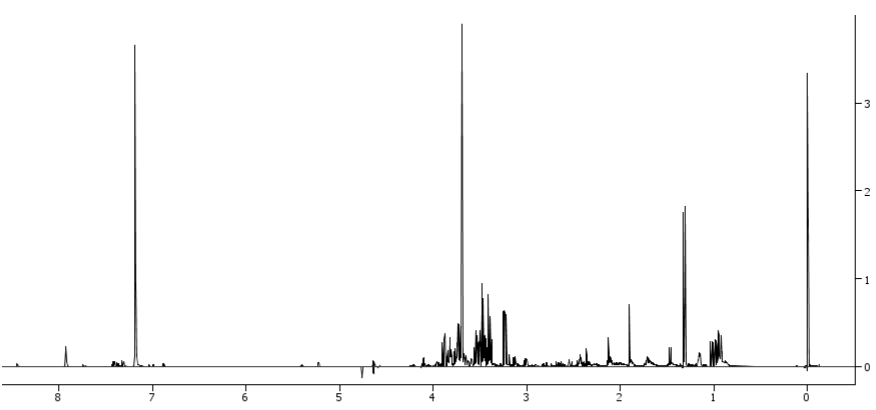
Chapter 3

Metabolic alterations induced by virus infection



This chapter was based on the following manuscript:

Impact of Adenovirus infection in host cells metabolism evaluated by ^{1}H-NMR spectroscopy, Silva A.C., Teixeira P. A. and Alves P. M. (*submitted*)

Abstract

Adenoviruses are powerful vehicles for gene transfer vaccination and gene therapy applications. Although highly exploited in clinical settings, key aspects of adenoviral biology are still to be disclosed. Importantly, the adenoviruses subversion of host cell metabolism to fuel their own replication is not clear. The aim of this work was to gain insights on the metabolism of two human cell lines (HEK293 cells and an amniocyte-derived cell line, 1G3) after infection and replication of an adenoviral vector (AdV5) commonly used in gene therapy and vaccine clinical trials. We used ^{1}H-NMR spectroscopy, which allowed the quantification 35 metabolites in cell culture supernatants, with low sample preparation in a relative short time. The metabolic profiling identified significant differences between both cell lines in non-infected cultures, namely in glutamine and acetate metabolism, as well as by-product secretion. The main response to adenoviral infection was the increased glucose consumption and lactate production rates. Infected cultures, with and without glutamine supplementation, confirmed the exhaustion of this amino acid as one of the main causes of lower adenovirus production at high cell densities (10- and 1.5-fold less infectious particles per cell, in HEK293 cells and 1G3 cells, respectively). Moreover, different degrees of glutamine dependency for each cell line were highlighted. Overall, the observed changes in amino acids metabolism and by-products formation during infection were mapped, which can be useful for targeted bioprocess optimization.

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1. Introduction

Recombinant adenoviral vectors (AdV) with E1 gene deletion are amongst the most efficient viral vectors for gene delivery, both *in vivo* and *in vitro*, and have been the choice in several therapeutic and vaccination applications. Production of E1-deleted human AdVs is usually performed in host cell lines that express the adenovirus E1A and E1B genes, being HEK293 host cell line of choice [1, 2]. However, safety issues concerning HEK293 cells tumorigenicity and their propensity to produce replication competent adenoviruses (RCAs) have arisen [3]. To obviate this, alternative producer cell lines were established, including the 1G3 cell line derived from human amniocytes, which was transformed with a plasmid containing the Ad5 E1A and E1B genes plus modified sequences from the pIX gene. These cells contain no overlapping sequences with AdV type 5 (AdV5), minimizing or eliminating the probability of generating RCAs [4].

A bottleneck that has been recognized for several years is the decrease of adenovirus productivity after infecting HEK293 or PER.C6 cells at cell concentrations higher than 1×10^6 cell/mL, resulting not only in low cell-specific product yields, but also considerably lower volumetric titers [5-7]. Most attempts to overcome this cell density effect in adenovirus production consisted in supplementing the culture medium with glucose, glutamine, a range of different complex nutrient mixtures, or performing complete or partial medium exchange at infection [8-12]. Strikingly, Adenovirus replication in the 1G3 cell line is less hampered at high cell concentration infection than in HEK293 cells [4]. Thus, a better understanding of how the infection process impacts the metabolism of producer cells would give us important information to increase productivity in high cell density cultures.

It has been reported that viral replication induces major changes in cell physiology and metabolism [13]. The metabolic efficiency to supply precursors for biosynthesis was proven to impact the synthesis of viral components, their assembly, release and final production levels [13, 14]. However, the metabolic characterization of AdV production processes has been mostly restricted to the analysis of the main extracellular metabolites, such as glucose, lactate, ammonia and glutamine [9, 15, 16]. While these studies provide an overview of cell metabolism, a larger picture of the overall metabolic state is required for

understanding the key cellular mechanisms operating during growth and virus production.

In this work, we applied ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy to generate data that permits a comparison of the exometabolomes of 1G3 and HEK293 cells, during growth and upon adenovirus infection at different cell concentrations. ^{1}H NMR allows profiling a wide range of compounds in the culture supernatant, while requiring minimal sample preparation and providing high reproducibility in a relatively short time.

2. Materials and methods

2.1. Cell lines and cell culture

HEK293 cells (ATCC CRL-1573 , adapted to grow in single cell suspension) and 1G3 cells [4] were routinely cultured in serum free Ex-Cell™ medium (Sigma-Aldrich, USA) supplemented with 4 mM glutamine (Invitrogen, UK), using 500 mL shake flasks (80 mL working volume). Cultures were seeded at a cell concentration of 0.5×10^6 cell/mL and were maintained on an orbital shaker at 130 rpm and incubated in humidified conditions at 37°C with 8% CO_2 in air.

2.2. Virus stock preparation and titration

A recombinant E1-deleted AdV type 5 expressing the Green Fluorescence Protein (AdV5-GFP) was used in this work. The AdV5-GFP virus was amplified in HEK293 cells cultured in monolayers using T-flasks and purified by CsCl gradients. AdV titration was performed by monitoring the expression of GFP reporter gene after infection of HEK293 cells seeded in 24-well plates, as previously described [17]. Briefly, cells were seeded at 0.25×10^6 cell/well and infected after 24 hours with 1 mL of serial dilutions of the AdV samples in DMEM with 10% (v/v) FBS. Cells were trypsin-detached at 17–20 hours post-infection (hpi) and the percentage of GFP-positive cells determined by flow cytometry (CyFlow Space, Partec, Germany).

2.3. Viral replication in shake flask and bioreactor cultures

1G3 cells and HEK293 cells were grown in shake flasks or stirred tank bioreactors with an inoculum of $0.5x10^6$ cell/mL. Shake flasks cultures were performed in 500 mL shake flasks (80 mL of working volume) that were maintained in an orbital shaker at 130 rpm in humidified atmosphere at 37°C with 8% CO_2 in air. Bioreactor cultures were performed with 1L working volume in Biostat DCU-I 2-L vessels (Sartorius Stedium, Biotech GmbH) equipped with rushton impellers. The temperature was settled and controlled at 37ºC. pO_2 was controlled at 50% air saturation with constant airflow (0.01 L/min) by sequentially varying the nitrogen partial pressure, agitation rate and oxygen partial pressure. pH was controlled at 7.2 by aeration with a CO_2 gas-mixture and 1M $NaHCO_3$.Once cells reached a concentration of 1 and/or 3 x 10^6 cell/mL, cultures were infected with a multiplicity of infection (MOI) of 5 infectious particles (ip) *per* cell. For virus infections experiments at CCI $3x10^6$ cell/mL in bioreactor, glutamine was added at 48 h after inoculation to a final concentration of 5 mM. Samples were collected along culture time for cell counting. Supernatant samples were clarified at 1000 g for 10 min and stored at -20ºC for metabolome analysis. Virus production was assessed at 48 hpi. Culture samples were centrifuged at 1000 g for 10 min at 4ºC and the supernatants kept at -85ºC for titration of extracellular virus. In order to quantify intracellular virus content, cell pellets were ressuspended in 10 mM Tris buffer at pH 8.0, supplemented with 2 mM $MgCl_2$ (Merck, Darmstadt, Germany) and 0.1% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO). Cells were disrupted by vortexing for 1 min and debris removed by centrifugation at 3000 g for 10 min at 4ºC. The resulting cell lysate was dispensed into small aliquots and stored at -85ºC, until further use.

2.4. Cell concentration and viability determination

Cell concentration and viability were determined by counting the cells on a haemocytometer using the Trypan Blue dye exclusion method.

2.5. Exometabolome Analysis

^{1}H-NMR was performed in a 500MHz Avance spectrometer (Bruker, Billerica, MA) equipped with a 5-mm QXI inversed probe. Spectra were acquired using a NOESY-based pulse sequence with water pre-saturation, performing 256 scans with 4 s acquisition time, 1 s relaxation delay and 100 ms mixing time at 25ºC. DSS-d6 (Cat.No. 613150, Isotec, Sigma-Aldrich, St. Louis, MO) was used as internal standard for metabolite quantification in all samples. Samples were mixed with phosphate buffer (pH 7.4) prepared in D_2O (Cat.No. 151882, Sigma-Aldrich, St. Louis, MO) at a 2:1 ratio to minimize pH shifts. Before spectra acquisition, the spectrometer was calibrated by determining the 90º pulse and the water chemical shift center of each sample.

Each spectrum was phased, baseline corrected and integrated using Chenomx NMR Suite 7.1 (Chenomx, Inc., Edmonton, Alberta, Canada). Most metabolites are defined by several clusters at different chemical shifts, which in some cases may overlap or be affected by the damping effect caused by water suppression, inducing an underestimation of their concentrations. Therefore, after automatic fitting of each metabolite, the best resolved and farthest peak from the water region was chosen for manual adjustment and metabolite quantification. This method allowed accurate quantification of 35 metabolites. A detailed explanation of the method developed and Chemical shifts of clusters used for quantification, errors and limits is reported in Duarte et al. (2014) [18].

Table 3. 1: List of metabolites identified and quantified by 1H-NMR.

Metabolites identified and quantified by ^{1}H-NMR			
Glucose	Histidine	Tryptophan	Glycerol
Lactate	Isoleucine	Tyrosine	Hypoxanthine
Glutamine	Leucine	Valine	Isobutyrate
Glutamate	Lysine	Acetate	Glyc-3-PC
Alanine	Methionine	Choline	Phosphocholine
Arginine	Phenylalanine	Citrate	Pyruvate
Asparagine	Proline	Ethanolamine	Succinate
Aspartate	Serine	Formate	Thymidine
Glycine	Threonine	Fumarate	

3. Results

3.1. Exometabolome analysis during growth of HEK293 and 1G3 cells

HEK293 and 1G3 cells were grown in the same serum-free media and the supernatant composition analyzed along culture time using ^{1}H-NMR. The final goal was to detect and quantify the highest amount possible of extracellular metabolites, in order to obtain an extended overview of both cells exometabolomes (**Figure 3. 1**).

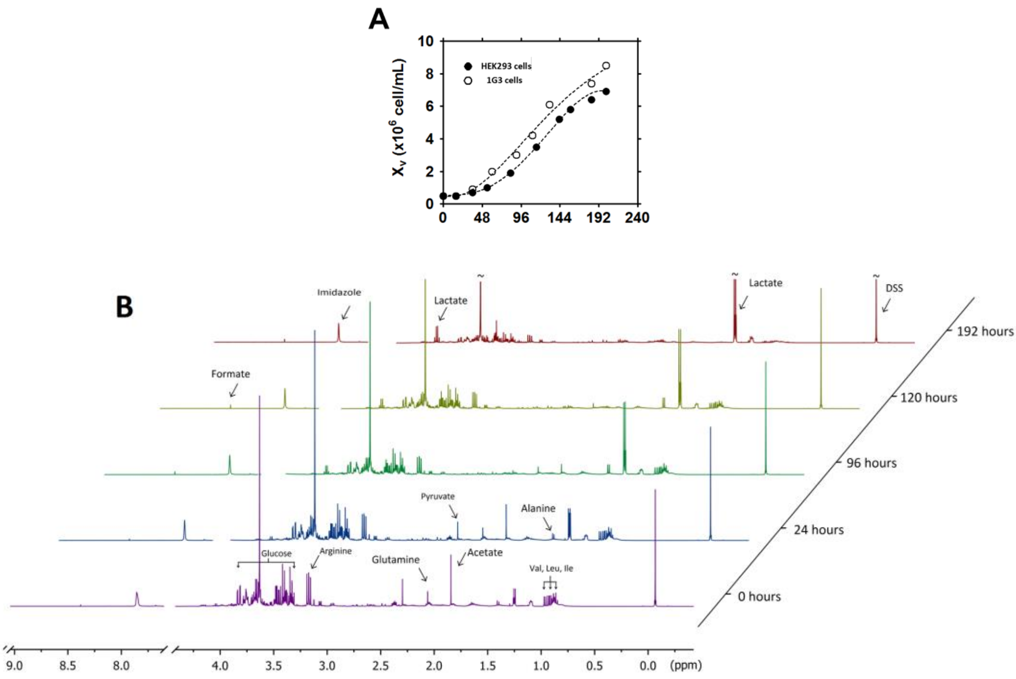

Figure 3. 1: **A**) Profiles of 1G3 and HEK293 cells growth cultured in shake flasks. **B**) Evolution of the ^{1}H-NMR spectrum of the supernatant over culture time (0h, 24h, 96h, 120h and 192h) for HEK293 cells. (Xv – viable cell concentration; DSS – internal standard for metabolite quantification).

Besides the main substrates (glucose and amino acids) and lactate, normally analyzed in mammalian cell cultures, it was possible to quantify pyruvate, acetate, the phospholipid precursors choline and ethanolamine, the nucleotide precursors hypoxanthine and thymidine, as well as several by-products (**Figure 3. 2, Table 3. 1**).

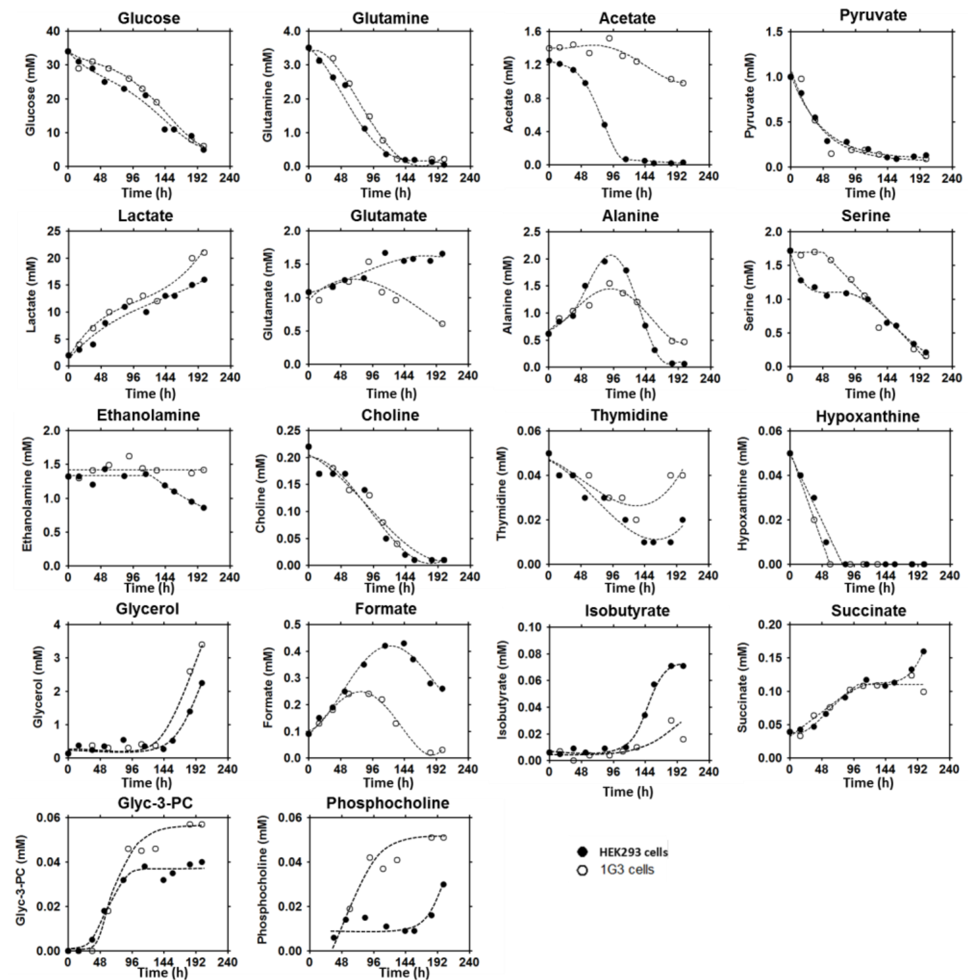

Figure 3. 2: Concentration profiles of selected metabolites in shake flask cultures of 1G3 and HEK293 cells.

Despite the similar growth profiles of HEK293 and 1G3 cells (**Figure 3. 1A**), the concentration profiles of specific metabolites were markedly different (**Figure 3. 2**). For instance, when glutamine is above 1 mM in the medium, HEK293 cells secrete alanine at higher rates than 1G3 cells. When glutamine is limiting, 1G3 cells start to consume glutamate and alanine, while HEK293 cells do not consume glutamate but take up alanine at higher rates. Another interesting difference between these cell lines concerns acetate, which was avidly uptaken by HEK293 cells until depletion, while 1G3 cells consumed it at a lower rate and only during the later phase of growth.

Pyruvate uptake was similar for the two cell lines, and was higher during the first 96 hours of culture (**Figure 3. 2**). Choline and ethanolamine are part of the

medium composition but both cell lines consume preferentially choline as precursor of cell membrane phospholipids. At 144 hours of culture, choline concentration was exhausted and HEK293 cells, but not 1G3, started to take up ethanolamine. The nucleotide precursor hypoxanthine was depleted very early in both cultures, and thymidine was more rapidly consumed by HEK293 cells.

Regarding metabolite secretion, lactate was the main by-product, being secreted at high levels in both cell lines, but other metabolites were also accumulated. Glycerol and isobutyrate also accumulated in the supernatant towards the end of the growth phase in both cell cultures. Formate was secreted until 72 hours of culture in the case of 1G3 cells and at a higher rate until 144 hours for HEK293 cells, following which it started to be consumed. The TCA cycle intermediates, like succinate, were also secreted to the supernatant. Finally, phosphocholine and glycerol-3-phosphocholine (Glyc-3-PC), both by-products from choline metabolism, were detected in increasing amounts after the initial 48 hours of culture (**Figure 3. 2**).

Overall, the results show that these human cell lines, derived from different tissues, exhibit several metabolic differences when cultured in the same medium. In particular, they respond differently to exhaustion of glutamine, consuming or not some medium components, and secrete at different rates by-products.

3.2. Impact of adenovirus infection

To evaluate the impact of adenovirus infection at the exometabolome level, 1G3 and HEK293 cells were infected with AdV5-GFP and supernatant samples were collected over time and analysed by ^{1}H-NMR. Both cell lines were infected at two different cell concentrations (1 and 3x10^{6} cell/mL) with a MOI of 5 ip/cell. The *per* cell productivity was lower when infection was performed at high cell concentration (**Table 3. 2**). Productivity levels for 1G3 cells decreased from 375 to 268 ip/cell, but still correspond to a 2.3-fold increase in volumetric titer. In the case of HEK293 cells, productivity levels presented a more pronounced decrease from 605 to 35 ip/cell, corresponding to 16-fold less AdV5-GFP per cell when were infected at CCI3.

Table 3. 2: Volumetric (ip/mL) and specific (ip/cell) adenovirus yields at 48 hpi in shake flask for 1G3 and HEK293 cells at CCI 1 or 3x10⁶ cell/mL.

	1G3 cells			HEK293 cells		
	CCI1	CCI3	CCI3/CCI1	CCI1	CCI3	CCI3/CCI1
ip/mL	3.8×10^8	8.6×10^8	2.3	6.1×10^8	1.3×10^8	0.2
ip/cell	375	268	0.7	605	37	0.1

Metabolite uptake and secretion rates were determined during the early phase of infection (from 0 to 24 hpi; **Figure 3. 3**). As a major response to adenovirus infection, both cell lines increased consumption of glucose (on average 2-fold) and secretion of lactate (up to 4-fold). Although these rates decreased at high CCI, their up-regulation upon infection could still be observed. Concerning glutamine, HEK293 cells consumed this amino acid at higher rates than 1G3 cells, as observed under non-infected conditions. Alanine secretion/consumption was not affected by adenovirus infection: at low cell densities, HEK293 cells produced alanine at higher rates than 1G3 cells, while at high cell concentrations HEK293 cells consumed alanine at higher rates than 1G3 cells. Glutamate secretion increased over 3-fold in infected cultures. Choline consumption increased upon infection at CCI1, while at CCI3 remained similar to non-infected conditions for both cell lines.

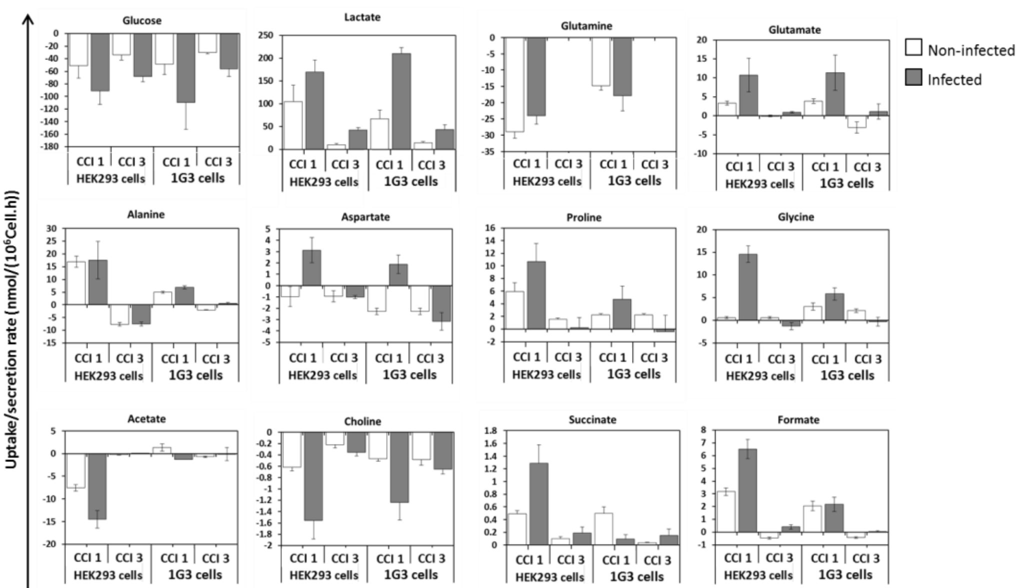

Figure 3. 3: Specific uptake/secretion rates of metabolites in non-infected and infected shake flask cultures, at CCI1 and CCI3. Specific rates were calculated by linear fit of extracellular metabolite concentrations *vs.* the integral of cell number. Negative values represent consumption and positive values represent secretion of metabolites.

Analysis of other metabolites further pointed out interesting differences in the response of each cell line to adenovirus infection. For instance, acetate consumption and formate secretion increased upon infection of HEK293 cells at low CCIs but not for 1G3 cells. Succinate secretion increased in infected cultures of HEK293 cells at CCI1, while it decreased for 1G3 cells. No other relevant changes were observed.

3.3. Impact of adenovirus infection in bioreactor cultures with glutamine re-feed

Next, we evaluated the impact of adenovirus infection at low and high cell densities under oxygen and pH controlled conditions (**Figure 3.4A**). To avoid glutamine exhaustion, as observed in shake flask cultures at CCI3, the concentration of this amino acid was replenished to 5 mM, 24 hours prior infection (**Figure 3.4B**). Infections performed at CCI1 produced over 1.5-fold higher ip's per cell than in shake flask cultures (**Figure 3.4C**). Moreover, a cell density effect on adenovirus replication was not observed for 1G3 cells, while for HEK293 cells the *per* cell productivity decreased to half when infection was performed at CCI3 compared to CCI1.

The culture media composition was analyzed along culture time and specific uptake/secretion rates were determined for non-infected and infected bioreactor cultures (**Figure 3.4D**). The two cell lines displayed different rates of glucose consumption and lactate production in non-infected cultures (around 2-fold-higher for 1G3 cells). Upon infection, these rates were up-regulated by a factor of 3 for HEK293 and 1.5 for 1G3 cells at CCI1 and with less extent for both cell lines at CCI 3. With regard to glutamine consumption, 1G3 cells maintained the same uptake rate in non-infected and infected cultures at both CCI's, while HEK293 cells increased glutamine consumption by 2-fold upon infection (as opposed to what was observed in shake flask cultures). Alanine secretion was higher for HEK293 cells, increasing by 2-fold at CCI1 and decreasing 3-fold at CCI3 compared to non-infected conditions. By turn, 1G3 cells kept the same alanine secretion rate in non-infected and infected cultures (as observed in shake flask cultures) (**Figure 3.4D**).

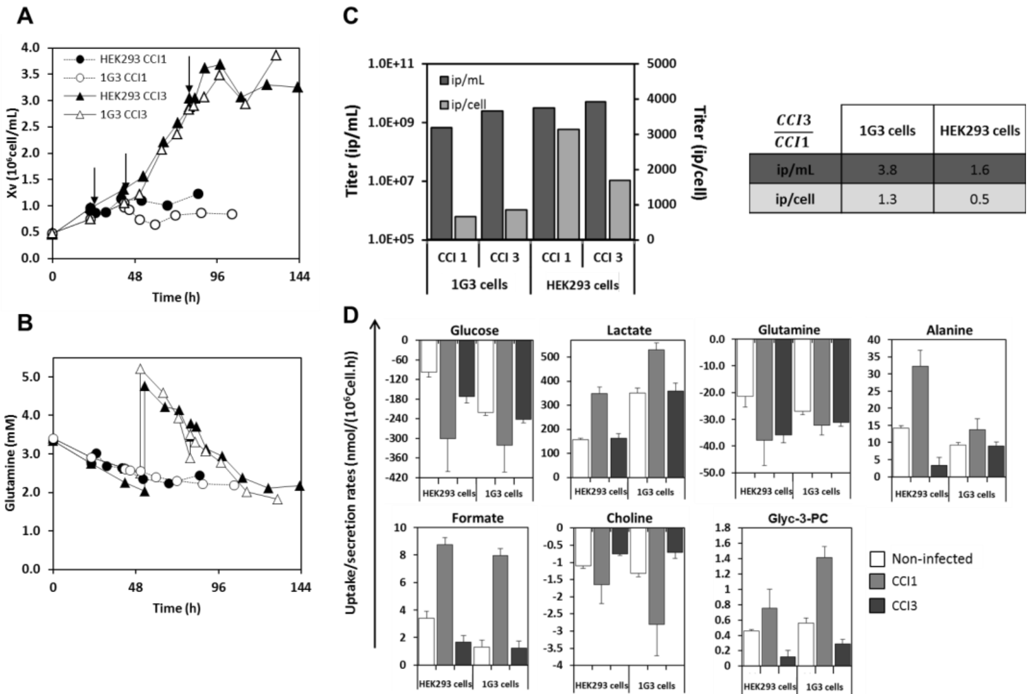

Figure 3. 4: Adenovirus replication of 1G3 and HEK293 cells in bioreactor cultures infected with AdV5-GFP at CCI 1 or 3x10^6 cell/mL. **A)** Viable cell concentration profiles (Xv). The arrows represent the time of infection (CCI 1 and CCI 3 and the time of glutamine re-feed). **B)** Profiles of glutamine concentration. **C)** Volumetric (ip/mL) and specific (ip/cell) AdV5-GFP yields at 48 hpi and titers normalized to CCI1. **D)** Specific uptake/secretion rates of metabolites in non-infected and infected bioreactor cultures. Specific rates were calculated by linear fit of extracellular metabolite concentrations *vs.* the integral of cell number. Negative values represent consumption and positive values represent secretion of metabolites.

Formate secretion increased in infected cultures at CCI1 for both cell lines in bioreactors, while in shake flask cultures, an increase was only observed for HEK293 cells. Also, choline consumption and glycerol-3-phosphate secretion increased upon infection at CCI1 and decreased at CCI3 for both cell lines.

4. Discussion

Viruses require host cellular metabolism to provide the energy and molecular building blocks needed for successful replication. In this study, we resorted to ^{1}H-NMR spectroscopy to identify metabolic differences between two human cell lines, 1G3 and HEK293, in scenarios of growth and adenovirus infection at low and high cell densities. It was possible to detect and quantify

changes in culture media composition throughout culture time, including metabolites that are not normally analysed in mammalian cells cultures. More specifically, we could identify and precisely quantify 35 metabolites, such as acetate, pyruvate, formate, TCA cycle intermediates, amino acids, lipid and nucleotide precursors.

Glutamine was the most consumed amino acid by both 1G3 and HEK293 cells, however HEK293 cells consumed glutamine and secreted alanine at higher rates than 1G3 cells. Moreover, when glutamine was at limiting levels, HEK293 cells consumed alanine at higher rates, but 1G3 cells started to take up glutamate in addition to alanine. It has been shown that adapting HEK293 cells to grow in glutamate supplemented medium instead of glutamine could contribute for an increase in AdV productivity [8].

Another interesting difference was the acetate consumption observed by HEK293 cells, but not in 1G3 cells. The principal entry point of acetate into mammalian metabolic network is by conversion to acetyl-CoA by the cytosolic enzyme acetyl-CoA synthetase (ACS) [19-21]. Acetyl-CoA then enters the TCA cycle by condensing with oxaloacetate to form citrate, or can be used for fatty acid synthesis and protein acetylation [22]. It has been reported that the activity of ACS varies from tissue to tissue and that kidney, from where HEK293 cells are derived, is one of the tissues with higher activity [23, 24]. Indeed, the different metabolic signatures of 1G3 and HEK293 cells are certainly related with their different origins. Several human cell lines from different tissues, including HEK293 cells, have been proteomically profiled revealing differences in enzyme levels from multiple metabolic pathways, including fatty acid metabolism, amino acid metabolism and glutathione metabolism [25].

Hypoxanthine and thymidine, nucleotide precursors, were metabolites identified that were consumed rapidly by both cell lines. Although not essential, it has been reported that these two metabolites contribute to decrease the lag phase regardless of the seeding concentration by stimulating the initial growth rate [26]. Glycerol and isobutyrate were also accumulated in the supernatant towards the end of the growth phase in both cell cultures. The accumulation of these metabolites has been also reported for CHO cells [27]. The TCA cycle intermediate succinate was also secreted to the supernatant, as reported for MDCK cells [28] and CHO cells [27, 29, 30].

The metabolic response of both cell lines to adenovirus infection was assessed by performing synchronous infections (MOI 5) at 1 and 3×10^6 cell/ml using two culture systems, shake flasks and bioreactors. Bioreactors were used to make sure pH was not affecting AdV productivity and since it is known that values lower than 7.2 affect productivity [31, 32]. Besides being a good down scale model for several process parameters, the use of shake flask do not allow pH control and we have previously showed that HEK293 and 1G3 cell cultures in this conditions can achieve pH's lowers than 6.5 [4]. Regardless of the culture system, cell line and CCI, glucose consumption and lactate production rates were up-regulated upon adenoviral infection. Most viruses induce aerobic glycolysis (also known as the Warburg effect) in the host cell metabolism [33, 34], including human cytomegalovirus [35], hepatis C virus [36] and Dengue virus [37], among others [34]. It was recently shown that the expression of the viral gene E4ORF1 is necessary for adenovirus induced upregulation of glycolysis by activation of Myc and allow maximal adenovirus replication [38].

An important aspect of viral vector production is the so-called cell density effect on infection, described for adeno [7, 8, 39] and other viruses [40]. Here, adenovirus replication was negatively impacted at high CCI and the decrease in productivity was much higher for HEK293 cells [4]. The use of controlled bioreactors and glutamine replenishing allowed an increase in AdV productivities at CCI3, bringing the specific yields closer to those obtained at low CCIs. In these conditions, 1G3 cells maintained the same glutamine uptake rate in non-infected and infected cultures, while HEK293 cells increased glutamine consumption by 2-fold upon infection at both CCIs. These results confirmed that 1G3 cells are less dependent on glutamine metabolism than HEK293 cells, as previously reported [4]. This characteristic has also been described for PER.C6 cells [41]. Since glucose carbon is shunt into lactate production as well as fatty acid synthesis, glutamine is required as an anaplerotic substrate to fuel the TCA cycle. A number of viruses have also been shown to require glutamine for replication [34], such as polioviruses [42], human cytomegalovirus [43] and vaccinia viruses [44]. Interestingly, a metabolomics study of primary HFF cells infected with vaccinia virus showed that infection increases intracellular glutamine and glutamate levels but does not increase glycolytic metabolites [44].

From the comprehensive analysis of medium composition performed in this work, only acetate (besides glutamine) was depleted in HEK293 cell cultures infected at CCI3. Therefore, the replication of adenovirus in this cell line at high cell densities should be examined with acetate supplementation. Overall, our systematic study using ^{1}H-NMR showed that this technique can contribute to a better understanding of the metabolic needs for adenoviruses production, and permitted to highlight a number of metabolic differences of two human producer cell lines. ^{1}H-NMR has been also applied to study the impact of echovirus infection [45], the effect of productivity enhancers on the metabolism of CHO cells [27], or to characterize the composition of conditioned medium in stem cell cultures [46].

Future studies should focus on carbon labelling experiments to follow the incorporation of tracer substrates into the different metabolic pathways recruited by infection. This will allow a more detailed metabolic signature of adenovirus infection, and help to devise cell and/or process engineering strategies like more specific fed-batch supplementations besides glucose and glutamine that will help to improve adenovirus production.

5. Acknowledgments

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6. Author contribution

Ana Carina Silva elaborated the experimental setup and design, performed the experiments, analyzed the data and wrote the chapter.

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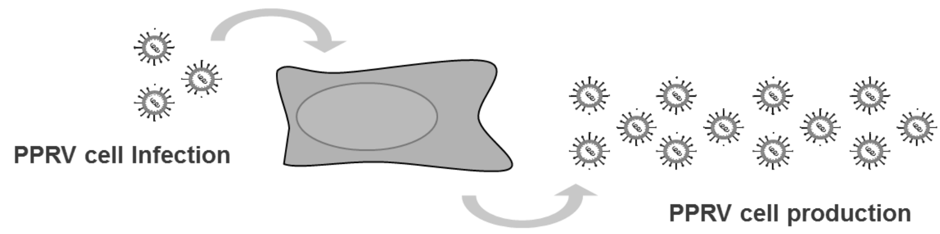
Chapter 4

Scalable culture systems using different cell lines for the Production of Peste des Petits Ruminants Vaccine



This part was based on the following manuscript:

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Abstract

Peste des Petits Ruminants (PPR) is considered as one of the major constraints to the productivity of small ruminants in Africa and Asian countries. Currently PPR control is done by vaccination with an attenuated PPR strain (Nigeria 75/1) produced in monolayers of Vero cells grown in roller bottles or static flasks.

This work focuses on the production of a PPR vaccine strain using stirred conditions as an advanced option for process scale-up. Non-porous microcarriers (Cytodex-1®) were used to support Vero cell growth in suspension cultures. The use of Ex-Cell™ medium could improve cell specific productivities obtained with standard serum containing medium, independently of the type of system used, i.e. static as well as suspension stirred cultures.

As an alternative, several cell lines adapted to grow as single cells in suspension (CHO-K1, BHK-21A and HEK293) and another anchorage dependent (MRC-5) were evaluated in their capacity to produce a PPR vaccine. BHK-21A and HEK293 cells grown as single cell suspension in serum-free medium were both suited to produce PPR vaccine with productivities similar to Vero cells, namely 10^6 $TCID_{50}$/ml. However, for the HEK293 cells, these results were only obtained 2-3 days later. CHO-K1 and MRC-5 cells have shown not to be suitable to adequately produce this virus.

These results provide further insights into the feasibility of applying microcarrier cell culture technology to produce PPR vaccine in Vero cells as well as in the alternative use of single cell suspension cultures of BHK-21A, significantly simplifying the existing production process.

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1. INTRODUCTION

Peste des Petits Ruminants (PPR) is an acute, highly contagious and fatal disease of sheep and goats and is considered as one of the major constraints to the productivity of small ruminants in Africa and Asian countries [1]. The causative agent, PPR virus (PPRV), is a member of the genus Morbillivirus within the *Paramyxoviridae* family and is antigenically closely related to the Rinderpest (RP) virus [2], another member of the genus Morbillivirus that causes similar diseases in wild and domestic ruminants. The PPR disease is characterized by high fever, ocular and nasal discharges, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhea. The main characteristic of the pathogenesis of PPRV infection, as for all other Morbilliviruses, is the profound but transient immunosuppression induced by this virus in its host with the consequence of increased susceptibility to opportunistic infections and increased mortality. The PPR virus is enveloped, pleiomorphic and has a single-stranded RNA genome of negative-sense. It is composed of 15,948 nucleotides, the longest of all Morbillivirus genomes sequenced so far [3]. The viral replication occurs in the host cell cytoplasm and the virus is released by budding [4]. However, these mechanisms are not very efficient in this virus family and a substantial amount of the produced viruses are kept associated to the host cells membranes.

Until recently an heterologous Rinderpest vaccine has been used for the control of PPR [5]; however, due to the ongoing Rinderpest eradication programs, the use of this vaccine for the control of PPR has been restricted or banned to avoid complications in RP sero-surveillance. The need for a PPR homologous vaccine led to the development of an attenuated viral vaccine based on the strain PPR 75/1, isolated in Nigeria in 1975 [6]. This strain was attenuated by 74 serial passages in Vero cell cultures [5] and it is currently the only vaccine permitted for use in sheep and goats [7], the efficacy being demonstrated at a dose of 10^3 $TCID_{50}$ [8].

Nowadays this vaccine is produced in Vero cells using classical techniques, i.e in T-flasks or roller bottles. These strategies involve high efforts concerning consumables and have limited scalability, significantly increasing the

bioprocess costs. Thus there is a need for better vaccine production processes for controlling future PPR outbreaks.

Vero cells (from African green monkey kidney), together with other anchorage-dependent cell lines like Madin-Darby bovine or canine kidney (MDBK or MDCK) and human diploid lung fibroblast (MRC-5), have been traditionally used for viral vaccine production; however, being strictly anchorage dependent, these cells require the use of microcarrier technology for production in scalable stirred tanks. Bioprocesses using Vero cells grown in microcarriers have been reported for the production of amongst others, Rabies virus [9], Reovirus [10], Japanese encephalitis virus [11], with or without the use of a serum-free medium. Stirred tank bioreactors allow for a fine control of cell environment (nutrients, pH, O_2 and agitation) permitting different modes of operation (batch, fed-batch and perfusion). Nevertheless, the use of microcarriers not only significantly increases the cost of the process but also requires extra optimization steps during development (e.g. microcarrier type and concentration, optimum inoculation concentration, harvesting time) [9, 12, 13]. Thus, to avoid the use of microcarriers, some of the "more" industrial anchorage dependent cell lines (CHO, BHK and HEK293) have been adapted to grow as single cells in suspension [14-20], allowing for higher cell densities with concomitant increases in volumetric productivities and an easier scalability of the manufacturing processes. However, the use of these cell hosts for viral propagation is not straightforward: depending on the virus, host cell characteristics can affect viral infection and replication hampering either vaccine production and/or its efficacy.

Another critical aspect in the production of live-attenuated viral vaccines is the maintenance of the attenuation characteristics of the vaccine strain, i.e. ensuring that no reversion to virulence takes place during the propagation and production phases of the bioprocess. A strategy commonly used to maximize the maintenance of the attenuated phenotype is to decrease the number of virus amplification steps. Vaccine production should be based on the virus seed lot system and the working virus seed lot should not be more than five passages from the master virus seed lot [21].

It was recently reported that, for the Rinderpest (RP) virus vaccine strain, also attenuated by several passages in Vero cells, the attenuating mutations occur in most of its genes, none of which being sufficiently debilitating to

induce strong pressure for reversion [22]. In the case of the PPRV vaccine, the attenuated phenotype is not yet characterized and no data is available regarding the spontaneous reversion to virulence; due to the close relation to RP (same family and same genus) a similar behavior is expected.

In this work a cultivation system using Vero cells and microcarrier technology was established for producing a PPRV attenuated vaccine. In parallel, the permissibility of BHK-21A, CHO-K1, MRC-5 and HEK293 cells for PPRV production was evaluated. Finally, the possibility of producing this vaccine in a commercially available defined, serum-free medium was also assessed.

2. MATERIALS AND METHODS

2.1. Cells and Virus

Vero cells (ECACC, 84113001) were maintained in Minimum Essential Medium (MEM), supplemented with 2 mM glutamine and with 10% (v/v) Foetal Bovine Serum (FBS). BHK-21A cells (a subclone of ATCC accession CCL-10) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamine and 5% (v/v) FBS. CHO-K1 cells (ATCC, CCL-61) were maintained in DMEM/HAM's F12 containing 4 g/L glucose, 4 mM glutamine and 5% (v/v) FBS. MRC-5 cells (ECACC, 97112601) and HEK293 adherent cells (ATCC, CRL-1573) were maintained in MEM with 2 mM glutamine and supplemented with 5% (v/v) FBS. All media and supplements were from Gibco except the DMEM/HAM's F12 and the glucose, purchased from Sigma. All adherent cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. Suspension-adapted human HEK293 cells were grown in 125 ml Erlenmeyer flasks, with 40 mL CD293, a serum-free medium (Gibco) supplemented with 6 mM glutamine at 37ºC in a humidified atmosphere of 8% CO_2 in air, using orbital agitation (approx. 160 rpm). Routinely an inoculum of 0.5 x 10^6 cell/mL was used. Suspension-adapted human BHK-21A cells were grown in 125 ml Erlenmeyer flasks, with 35 mL SFM4CHO, a serum-free medium (Hyclone) with 35 mM glucose, 3.5 mM glutamine and 0.05% (w/v) Pluronic-F68 (Sigma), at 37ºC in a humidified atmosphere of 7% of CO_2 in air, using orbital agitation (approx. 110 rpm). Routinely an inoculum of 0.35 x 10^6 cell/mL was used.

The PPR vaccine strain used was PPRV (Nig 75/1), kindly provided by Dr. Geneviève Libeau (CIRAD-EMVT, France), at passage 75. This strain was routinely amplified in Vero cells and stored at -80ºC, using standard techniques [8]. The seed virus bank was prepared and stored in MEM medium with 2% (v/v) FBS with a titer of 1 x 10^6 $TCID_{50}$/mL, from passage 76 to 77 not exceeding 5 passages from the master seed bank of the vaccine, according to Diallo et al [5].

2.2. Production of PPRV in monolayer cultures

To evaluate the ability of different cell lines to produce PPRV, BHK-21A, MRC-5, CHO-K1, HEK293 and Vero cells were seeded at 0.1 x 10^6 cell/mL in 25 cm^2 T-flasks, to a final culture volume of 5 mL *per* flask. 24-hours (h) after inoculation the cells were infected with PPRV using a multiplicity of infection (MOI) of 0.1. The flasks were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in air. The cultures were checked daily for development of cytopathic effect (cpe). The same strategy was used to evaluate the effect of lower MOIs (0.01 and 0.001) in PPRV production in Vero, HEK293 and BHK-21A cells. To monitor the infection kinetics, one flask was harvested daily and its content (cells *plus* supernatant) stored at -80ºC prior to virus titration.

2.2.1. Effect of serum-free medium in PPRV production on Vero Cells

Vero cells were inoculated directly into VP-SFM (Gibco) or Ex-Cell™ for Vero (SAFC) (from this point onwards just referred as Ex-Cell™) serum-free medium supplemented with 4 mM glutamine from cells grown in MEM containing 10% (v/v) FBS after a centrifugation (10 min, 500 *g*) to remove the serum containing medium. The cells were seeded and infected 24 hours after inoculation using a MOI of 0.01, and were monitored as described above (2.2).

2.3. Production of PPRV in stirred cultures

2.3.1. Vero cells on microcarriers cultures

Cultures were carried out in 125-mL spinner vessels (Wheaton, Millville, USA) previously silanized with a solution of dimethyldichlorosilane (Merck, Darmstadt, Germany). Cytodex-1® microcarriers (MCs) (GE HealthCare, Sweden)

were prepared (3g/L) according to the manufacturer's instructions, and sterilized "in situ". Prior to use, the MCs were washed twice with pre-warmed culture medium and incubated for 15 minutes at 37ºC before cell inoculation. The cell inoculum was obtained from 175 cm^2 T-flasks (Nunc) with confluent Vero cells growing in MEM supplemented with 2 mM glutamine and 10% (v/v) FBS. The spinners were inoculated with 0.1 x 10^6 cell/mL and were maintained in a humidified atmosphere of 5% CO_2 in air at 37ºC. The cultures were stirred continuously at 50 rpm from the inset of inoculation. To evaluate the total cell number, an aliquot of 1mL of the culture suspension was washed once with phosphate buffered saline (PBS), then treated in 0.95 mL of 0.1M citric acid (Merck, Darmstadt, Germany) containing 0.1% (w/v) crystal violet solution (Merck, Darmstadt, Germany) and 1% (v/v) Triton X-100 (Sigma) and incubated at least 1 h at 37ºC. The released nuclei were counted using a Fuchs-Rosenthal counting chamber (Brand).

The specific growth rate, μ (h^{-1}) was estimated using the following equation:

$$\mu = \frac{LnX_n - LnX_{n-1}}{t_n - t_{n-1}} \quad (1)$$

where X represents the cell number per mL, t represents the time points of sampling expressed in hours (between 24 to 96 h that corresponds to the exponential phase of cell growth), the subscripts n and n-1 defining two succeeding sampling points.

The cell doubling time (t_d) was calculated using the trivial equation:

$$t_d = \frac{Ln(2)}{\mu} \quad (2)$$

2.3.1.1. Effect of MOI in PPRV production

Cells were inoculated as described above (2.3.1) with MEM medium supplemented with 10% (v/v) FBS. 24 h later the cells were infected with PPRV using MOI's of 0.1, 0.01 and 0.001. Samples were taken daily to perform cell counting and virus titration.

2.3.1.2. Effect of culture medium in PPRV production

Cells were inoculated as described in 2.3.1 with MEM medium supplemented with 2% (v/v) FBS or with Ex-Cell™ medium supplemented with 4 mM glutamine and with $CaCl_2$ to a final concentration of 200 mg/mL to improve cell attachment onto microcarriers. The inoculum was obtained from 175 cm^2 T-flasks (Nunc) with confluent Vero cells after centrifugation (10 minutes, 500 *g*) to change the MEM medium with 10% (v/v) FBS for the respective culture medium. 24 hours after inoculation the cells were infected with PPRV using a MOI of 0.01. At infection, in one of the experiments 90% of MEM medium with 2% (v/v) FBS was replaced by Ex-Cell™ Vero medium supplemented with 4mM glutamine. Samples were taken daily to perform cell counting and virus titration.

2.3.2. HEK293 and BHK-21A cells in single cell suspension cultures: Effect of MOI in PPRV production

HEK293 and BHK-21A cells were seeded routinely as described above (2.1) and 24 hours later were infected with a PPRV suspension using different MOIs namely, 0.1, 0.01 and 0.001. To monitor the infection kinetics 1 mL samples were taken daily and stored at -80ºC pending virus titration.

2.3.3. PPRV production in Vero cells and BHK-21A cells using STB

Bioreactor cultures were performed with 2L working volume in fully equipped Biostat DCU-I 2-L vessels (Sartorius Stedium, Biotech GmbH) with two 6-blade Rushton impellers for BHK-21A cells or two 3-blade impellers for Vero cells in microcarriers. The temperature was settled and controlled at 37ºC. pO_2 was controlled with constant airflow by varying sequentially the nitrogen and oxygen partial pressures in gas inlet. For BHK-21A cells, pO_2 was controlled at 30% air saturation with an agitation rate of 60 rpm (0.02 L/min with ring sparger) and for Vero cells pO_2 was controlled at 40% air saturation with an agitation rate of 50 rpm (0.1 L/min by headspace). pH was controlled at 7.2 by aeration with a CO_2 gas-mixture and 1M NaHCO3.

Vero cells inoculum was obtained from 175 cm^2 T-flasks (Nunc) with confluent cells grown in MEM supplemented with 2 mM glutamine and 10% (v/v) and the process was done using the same culture medium. Microcarriers were

treated as previously described in section 2.3.1 in the cell inoculation flask and the bioreactor vessel was also previously silanized. Before bioreactor inoculation, Vero cells (0.1 x 10^6 cell/mL) were allowed to slightly attach to the microcarriers before transfer to the bioreactor. BHK-21A cells were culture routinely as described above (2.1) and the bioreactor was perform with an inoculum of 0.35 x 10^6 cell/mL in serum-free medium. The cultures were infected at 24 hours-post inoculation with an MOI 0.01 for Vero cells and MOI 0.01 for BHK-21A cells. To monitor the infection kinetics 1 mL samples were taken daily and stored at -80ºC pending virus titration.

2.4. Virus Titration

Virus titration ($TCID_{50}$) was done with Vero cells in 96 well microtiter plates using standard cell culture procedures [8]. Briefly, 10-fold serial dilution of samples in MEM containing 10% (v/v) FBS were prepared. For each dilution 12 replicates were done with 100 μL virus suspension and 100 μL cell suspension (0.2x10^6 cell/mL) *per* well. All the test plates were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 in air. Cytopathic effect (cpe) was checked on day 12 of titration. End points ($TCID_{50}$/mL) were calculated according to the statistical method of Spearman-Karber [23], simplified for calculations in MS Excel format. All the samples were titrated in triplicate and the experiments were repeated at least twice. Each sample was freeze/thawed (1x) to ensure release of virus from cell debris and cell membranes. This procedure is required because in this viral family, the virus release mechanisms are not very efficient and part of the virus stays cell-associated. This procedure can increase the harvested virus production titer by up to 1 log [24].

3. RESULTS

3.1. PPRV production in Vero cells in static cultures

To assess the effect of the MOI in PPRV productivity using static conditions, Vero cells grown as monolayers were infected 24 hours post-inoculation with MOIs of 0.1, 0.01 and 0.001.

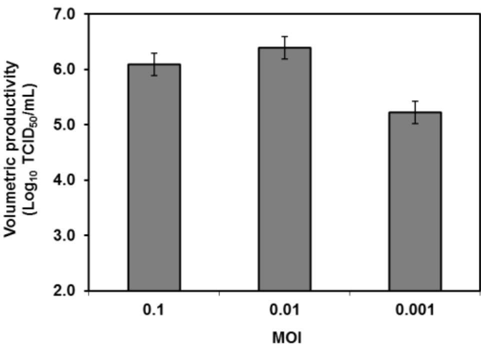


Figure 4. 1: Effect of MOI in PPRV production using monolayer cultures of Vero cells under static conditions with MEM supplemented with 10% (v/v) FBS. Error bars represent the standard error of triplicates.

The results, presented in **Figure 4. 1**, show that PPRV productivity was slightly higher (1.2 x 10^6 $TCID_{50}$/mL) when a MOI of 0.01 was used; the lower productivity being obtained for 0.001.

In order to select a serum-free medium for Vero cell growth and PPRV production, two different commercially available formulations, VP-SFM and Ex-Cell™ where tested for their ability to produce PPRV. Cells cultured using serum containing medium were collected, centrifuged and cultured directly into the serum-free medium; 24 hours post inoculation they were infected with a MOI of 0.01.

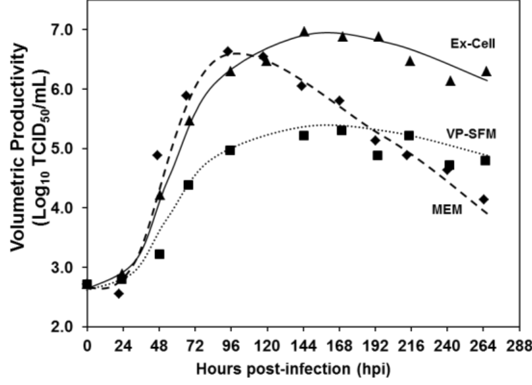


Figure 4. 2: Effect of serum-free media in the production of PPRV using monolayer cultures of Vero cells in static conditions (MOI of 0.01). MEM 10% FBS (◆); Ex-Cell™ (▲); VP-SFM (■). The lines are guides to follow the progression of the data.

Ex-Cell™ medium showed to be able to slightly increase the PPRV volumetric productivity comparing to the PPRV productivity obtained using the MEM medium (**Figure 4. 2**). The use of VP-SFM resulted in 1 log decrease in the PPRV productivity. The maximum cell density obtained was similar for all the culture media used; however, for the serum-free media such maximum was achieved 1 day later (data not shown) and thus the highest viral titer was obtained 1-2 days later than when MEM was used. The maximum viral titer obtained with the MEM medium was rapidly lost with increasing culture time showing that the virus is not very stable under these conditions. Although the maximum productivity is obtained at a later time, when serum-free media are used, the higher viral titers obtained could be maintained for longer periods of time.

3.2. PPRV production in Vero cells on microcarrier cultures

To evaluate the feasibility to produce PPRV in stirred tank bioreactors Vero cells were grown on Cytodex-1® microcarriers with MEM supplemented with 10% FBS and infected with different MOIs.

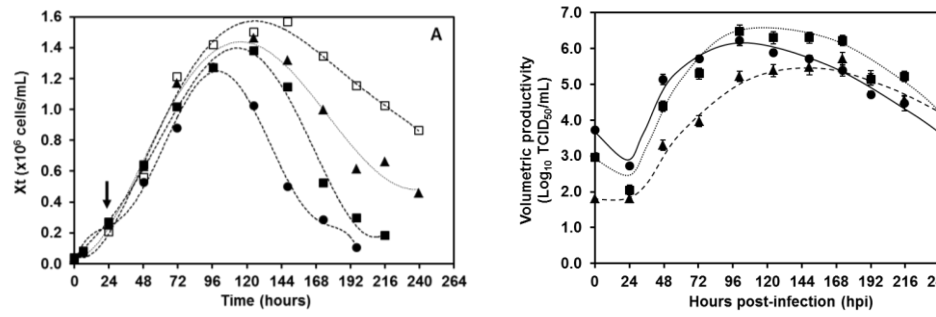


Figure 4. 3: Effect of MOI in cell growth (A) and PPRV production (B) by Vero cells grown on 3 g/L of Cytodex-1® in spinner flask in MEM medium supplemented with 10% (v/v) FBS. Control Cells (□); MOI 0.1 (●); MOI 0.01 (■); MOI 0.001 (▲); the arrow corresponds to time of infection. Error bars represent the standard error of triplicates and the lines are guides to follow the progression of the data.

Cell concentrations of cultures infected with decreasing MOIs reached increasingly higher maximum cell yields (**Figure 4. 3, A**). As expected, the cultures also attained their maximum cell yield at different times, as a function of the MOIs used. At MOIs of 0.1 and 0.01 the cells stopped growing and the cell concentration started to decrease at about 96 hours post-infection (hpi). At

the lowest MOI used (0.001) the maximum cell yield reached was slightly lower than that of the control. Cell growth rate was not affected after viral infection and was maintained at approx. 0.028 ± 0.004 h^{-1}, corresponding to a doubling time of approx. 25 h.

The volumetric PPRV titers are shown in **Figure 4. 3, B**. Cells infected at higher MOIs reached maximum titers earlier than the cultures infected at lower MOIs. Furthermore, for the lowest MOI, lower maximum viral yields were obtained. The cultures infected with the midrange MOI (0.01) led to the higher productivity with a $virus_{max}/cell_{max}$ of 2.1 ± 1.0 and a titer of 6.5 ± 0.5 (log_{10} $TCID_{50}$/mL) at 96 hpi. The lower MOIs tested allowed the maintenance of higher titers for longer whereas for the higher MOIs the maximum titers achieved were more rapidly lost along culture time.

3.2.1. Effect of culture media composition in PPRV production in Vero cells on microcarrier cultures

Figure 4 shows the effect of the culture media composition upon PPRV production in Vero cells grown on Cytodex-1® microcarriers. The maximum cell concentration was achieved for cells grown in MEM supplemented with 10% FBS (1.4 x 10^6 cell/mL). When the serum concentration was reduced to 2%, the specific cell growth rate was not affected (0.028 ± 0.004 h^{-1}) but the maximum cell concentration decreased to 0.6 x 10^6 cell/mL. The maximum cell density for cells grown in the Ex-Cell™ medium was lower (0.8-1.0 x 10^6 cell/mL) than for the cells grown in the 10% FBS containing medium.

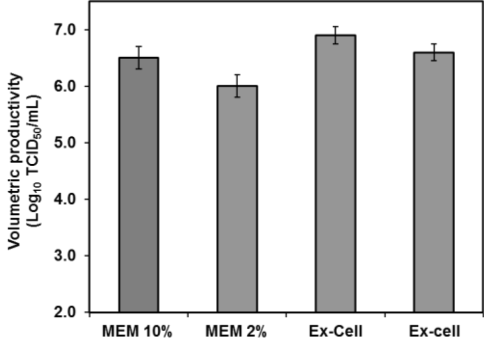


Figure 4. 4: PPRV productivity at a MOI of 0.01 from Vero cells grown in microcarrier cultures in different culture media. Ex-Cell™(ex) - Cells inoculation with MEM 2% and 90% medium exchange at infection to Ex-Cell™ medium. Error bars represent the standard error of triplicates.

Meanwhile, a decrease in viral yields was obtained when cells were grown in MEM with 2% FBS; the corresponding maximum titers were achieved at 96 hpi. After this period, and similarly to what was previously observed for static cultures (**Figure 4. 2**), a significant decrease in viral titer was observed for the remaining time of the culture. Although the maximum cell density was lower for the Ex-Cell™ medium than for the MEM medium with 10% FBS, viral volumetric productivity was comparable, depicting an increased cell specific productivity (7.5 ± 1.8 $virus_{max}/cell_{max}$ for Ex-Cell™ medium exchange at infection and 4.7 ± 1.6 $virus_{max}/cell_{max}$ for cells grown in Ex-Cell™). As for monolayer cultures grown in static conditions the higher titers were maintained for longer periods when Ex-Cell™ medium was used.

3.3. Evaluating different cell lines for the production of PPRV

The ability of five cell lines to support the production of PPRV was assessed in monolayer cultures grown in stationary conditions: two anchorage-dependent cell lines (i) Vero (the cell line normally used for the production of this vaccine) and MRC-5 and (ii) three cell lines adapted to grow as single cells in suspension, 293, BHK-21A, CHO-K1. The cells were infected at a MOI of 0.1 at 24 hours post cell inoculation, corresponding to a viral inoculum around 1×10^4 $TCID_{50}$/mL. The maximum PPRV titer obtained for each cell line is presented in **Figure 4.5**.

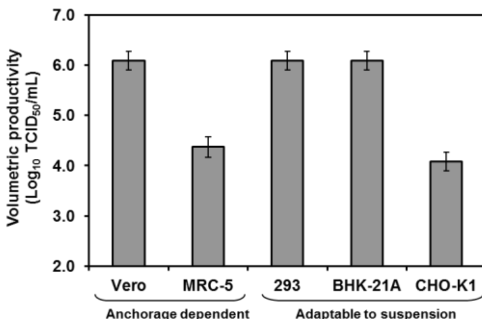
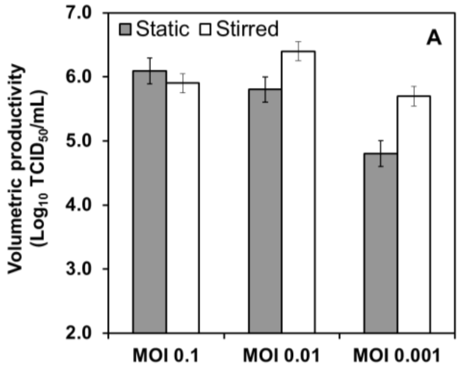


Figure 4.5: Effect of cell line in PPRV production using a MOI of 0.1 in Vero, MRC-5, HEK293, BHK-21A and CHO-K1 cells. (The data correspond to the maximal virus titer obtained and error bars represent the standard error of triplicates.)

The virus was able to replicate and the titer increased by at least 2 orders of magnitude for Vero, HEK293 and BHK-21A cells (approx. 1×10^6 $TCID_{50}$/mL). In

contrast, there was no amplification of virus in MRC-5 and CHO-K1 cells. The maximum volumetric PPRV productivity occurred after 96 hpi for Vero cells and BHK-21A. A delay of 24 hours was observed for HEK293 cells, where the maximum titer was achieved around 120 hpi.



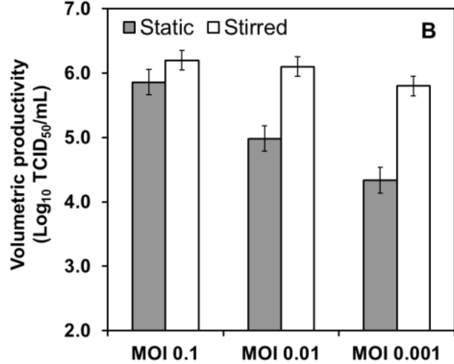


Figure 4. 6: Effect of MOI and culture system in PPRV production on HEK293 (A) and BHK-21A cells (B). Error bars represent the standard error of triplicates.

HEK293 cells are commonly used for viral production, namely for adenovirus [16, 17], its use for Rinderpest virus rescue from cloned cDNA having also been reported [25]. BHK cells have been used for decades in the production of the Foot and Mouth Disease vaccine [14] as well as for experimental rabies vaccine in suspension cultures [15]. As the results obtained show it is also possible to produce infectious PPRV in HEK293 cells and BHK-21A (**Figure 4.5**). Given their potential, these cell lines were selected for further studies regarding PPRV production. First, the effect of lower MOIs, namely 0.01 and 0.001, were tested. The results obtained showed that the productivity decreased for lower MOIs and the values obtained were somewhat than those obtained for Vero cells (**Figure 4.** 6, front bars).

3.4. PPRV production in single cell suspension cultures using HEK293 and BHK-21A cells

The feasibility of HEK293 and BHK-21A to grow as single cell suspensions in stirred tank bioreactors (STB) make them appealing alternatives for PPR production. The results obtained at different MOIs in stirred conditions are shown in **Figure 4.** 6 (back bars). Independently of the MOI tested after viral

infection, BHK-21A cells reached a maximum cell concentration of 2.3 x 10^6 cell/mL; HEK293 cells grew to a maximum of 3.5 and 2.9 x 10^6 cell/mL for the lower (0.001) and for the higher (0.1) MOIs tested, respectively. These values correspond to growth rates of 0.021 ± 0.002 h^{-1} for BHK-21A cells and 0.018 ± 0.001 h^{-1} for HEK293 cells. The maximum volumetric productivities were obtained for BHK-21A at 96 hpi (comparable to Vero cells grown on microcarriers) and for HEK293 cells 2-3 days later, i.e. 144 hpi. The different MOIs did not affect the final viral yields and the values were similar to those obtained for Vero cells, i.e. 10^6 $TCID_{50}$/mL.

Similar volumetric productivities were attained for Vero, BHK-21A and HEK293 cultures (**Figure 4.5, Figure 4.** 6). Since higher cell densities were achieved, it is obvious that HEK293 and BHK-21A cell specific productivities were lower (approx. 0.1-0.7 $virus_{max}/cell_{max}$) than those obtained for Vero (2.1 $virus_{max}/cell_{max}$).

3.5. PPRV production in STB using Vero cells and BHK-21A cells

To ensure that production of PPRV is possible in larger scales, the process was up-scale to 2 L for Vero and BHK-21A cells using the best MOI obtained for each cell line (MOI 0.01 and MOI 0.001, for Vero and BHK-21A cells respectively). Both cell lines presented similar growth profiles and PPR virus production than those presented at smaller scale (data not shown). The results obtained (**Figure 4. 7**) showed that it is possible to maintain PPR virus volumetric productivities in STB.

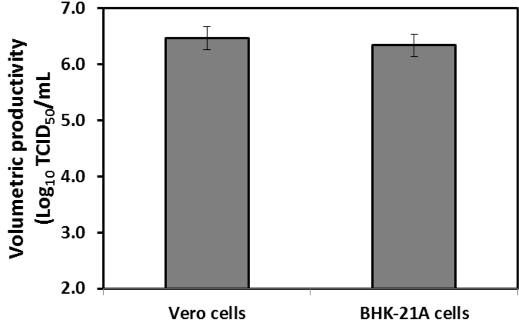


Figure 4. 7: PPRV production on Vero and BHK-21A cells in STB at 96 hours post infection. Error bars represent the standard error of triplicates.

4. DISCUSSION

Given the importance of controlling PPR outbreaks, the aim of this work was to evaluate alternative conditions for scalable production of a PPR attenuated vaccine instead of the currently available PPR vaccine produced in Vero cells using static culture systems (roller bottles and/or T-flasks). Our first strategy was to assess the feasibility of microcarriers technology, in particular Cytodex-1®, for PPRV production in stirred tank bioreactor using Vero cells; the use of serum-free formulations (Ex-Cell™) was also evaluated.

Cytodex-1® allowed for a maximum cell density of 1.5 x 10^6 cell/mL when MEM supplemented with 10% FBS was used (**Figure 4. 3**). These results are in agreement with previous reports by Appaiahgari et al. [26] using similar culture conditions and Mendonça et al. [27] using higher microcarriers concentration (10 g/L).

Regarding maximum cell concentration, one serum-free formulation (Ex-Cell™) allowed for higher cell densities than MEM containing 10% FBS medium (1 and 1.5 x 10^6 cell/mL, respectively). Maximum cell yields of 1 to 2 x 10^6 cell/mL were previously reported for microcarrier cultures of Vero cells using either serum-free or low serum medium formulations [9, 10, 28]; however, all these reports used higher inoculum concentrations denoting smaller cell expansion ratios. With respect to virus production, serum-free medium allowed for higher cell specific productivities. The low PPRV thermostability at 37ºC [29, 30] being a critical issue for bioprocess development, especially when serum containing medium is used, the fact that viral production titers could be maintained for longer periods of time at Ex-Cell™ medium (**Figure 4. 2**) is a good indication for enlargement of the bioreactor harvesting window. Moreover, bioreaction perfusion strategies previously reported to increase significantly rabies virus production in Vero cells [13, 25] could thus be attempted.

Although Vero cells cultivated using the microcarrier technology and the use of serum-free medium showed promising results for the larger scale of PPRV production the requirement for a solid matrix to support cell growth in bioreactors, make this a costly strategy.

The ability to produce PPRV using of alternative cells lines able to grow as single cells in suspension (BHK-21A, HEK293 and CHO-K1), was evaluated. Their performance was compared with Vero and MRC-5 (traditionally used for vaccine

production) cells. CHO-K1 and MRC-5 cells have proved not to be suitable for PPRV production, eventually due to the lack of cellular receptors necessary for the virus to infect them. The cellular receptors are one of the major determinants of the host range and tissue tropism of a virus. It is known that most of the Morbillivirus use signaling lymphocyte activation molecules (CD150) as cellular receptors [31, 32] and some cell culture adapted strains have the potential to replicate in different cell lines; however, few things are known specifically for PPRV.

Both BHK-21A and HEK293 were able to produce PPRV (**Figure 4.5**). In contrast to Vero, for the suspension cultures of BHK-21A and HEK293, the maximum volumetric titer was independent of the MOI and similar for all cell lines tested (Vero, BHK-21A and HEK293) (**Figure 4. 6**). This approach is an appealing alternative for virus production because an infection strategy based in a low MOIs overcome extra virus amplification steps, undesirable in industrial production, and minimize the virus passage effect. Similar conclusions were reported previously for other virus/host cells systems such as baculovirus/insect cells [33].

The lower cell specific productivities obtained for HEK293 and BHK-21A, when compared with Vero cells, can result on the "lack of adaptation steps" of the virus to these cell lines. Viral infection and replication kinetics depend significantly on the virus and the host cell characteristics. It has been reported in the literature that PPRV is especially suited to grow in Marmoset B95a Cells (adherent cell line derived from the Epstein-Barr virus-transformed marmoset B-lymphoblastoid cells). B95a cells are not suitable for vaccine production purposes but they can be used for rapid isolation and propagation of several Morbilliviruses [24, 34-36]. *In vitro,* the PPR cytophatic effect is detected much earlier in B95a than in Vero cells confirming the dependency of viral replication on the cell substrate. PPRV adaptation to Vero cells is characterized by its slow and low production yields [5]. The viral stock used in our work was always prepared in Vero cells.

Eventually, the PPRV productivities obtained for HEK293 or BHK-21A cells could be increased by a previous adaptation, by serial passage, of the virus to these cell substrates. However, the most important problem in preparing live-attenuated viral vaccines in different cell substrates is (i) retaining the attenuation phenotypes and (ii) not causing the reversion to virulence. To our

knowledge the attenuation markers from the PPRV vaccine are not yet characterized, thus it is not possible to evaluate if the markers are maintained when the cell substrate used for PPRV vaccine production is changed. It is not possible to know if a low number of passages (<5) will affect pathogenecity; however, during development of the live attenuated PPRV vaccine the strain was not attenuated until passage 20 in Vero cells and just became avirulent at passage 55 [5]. It was also reported for RP virus (RPV), the closely related virus of the PPRV family, that the high attenuation and stability of the current vaccine (RBOK, attenuated RPV vaccine strain) are due to the accumulation of a number of separate mutations, none of which is itself so sufficiently debilitating that there is strong selective pressure in favour of the revertant [22]. To ensure that the attenuation phenotype was kept as much as possible, we have propagated PPRV in Vero cells and the other cell lines were only used for the final production run. Since a low MOI can be applied in PPRV production in the HEK293 and BHK-21A from of a seed virus produced in Vero cells the current seed bank for vaccine production can be applied, minimizing the virus passage.

In conclusion, the comparison of Vero, HEK293 and BHK-21A cells showed that all these cell lines were capable of efficient virus amplification, reaching maximum volumetric titers of 10^6 virus *per* milliliter. However, faster viral replication was observed in Vero and BHK-21A cells, maximum titers being reached 2–3 days earlier than in HEK293 cells. Industrially, this difference in viral growth kinetics between cell lines improves process economics. This makes the BHK-21A cells culture in single cell suspension the best choice for PPRV production, as an alternative to the Vero cell culture system. For using as a live-attenuated vaccine the immunogenicity and attenuation stability needs to be evaluated; thus the next step towards this novel approach for a PPRV vaccine production process is to prove its effectiveness *in vivo* (animal trials).

The work described herein is the first report of a PPR candidate vaccine produced in suspension culture with serum-free medium. The results presented herein constitute valuable information for the development of a large-scale cell culture process for producing PPR vaccine.

5. ACKNOWLEDGMENTS

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6. AUTHOR CONTRIBUTION

Ana Carina Silva elaborated the experimental setup and design, performed the experiments, analyzed the data and wrote the chapter.

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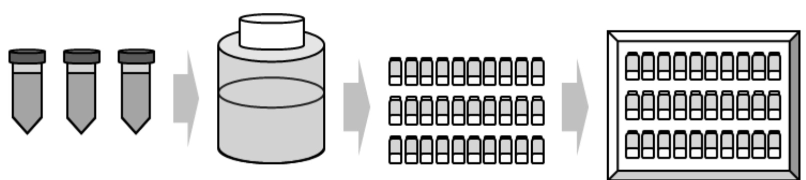
Chapter 5

Stabilization of biotherapeutics: from cell culture to final formulation



This chapter was based on the following manuscript:

Strategies for improved stability of Peste des Petits Ruminants Vaccine,

Silva, A.C., M.F.Q., Carrondo, M.J.T. and Alves, P.M., (2011) *Vaccine*, 29, 4983-4991.

Abstract

The main focus of this work was the improvement of the stability of the current PPRV vaccine. Firstly, new formulations based on the Tris buffer were tested, with and without the addition of sucrose and trehalose and compared with the formulation normally used to stabilize the vaccine, the Weybridge medium. The results show a virus half-life of 21 hours at 37ºC and 1 month at 4ºC for the Tris/trehalose liquid formulation and, in the lyophilized form, the formulation was able to maintain the viral titer above the $1x10^4$ $TCID_{50}$/mL (>10 doses/mL) for at least 21 months at 4ºC (0.6 log lost), 144 hours at 37ºC (0.6 log lost) and 120 hours at 45ºC (1 log lost).

Secondly, a strategy based on culture medium composition manipulation aiming at improving the intrinsic PPRV vaccine stability was also evaluated. The addition of 25 mM fructose resulted in a higher virus production (1 log increase) with higher stability (2.6 fold increase compared to glucose 25 mM) at 37ºC. Increased concentrations of NaCl, improved virus release, reducing the cell-associated fraction of the virus produced. Moreover this harvesting strategy is scalable and more suitable for a larger scale production than the freeze/thaw cycles normally used.

The information gathered in this work showed that it is possible for the PPRV vaccine to have adequate short-term stability at non-freezing temperatures to support manufacturing, short-term shipping and storage. The identification of a more stable formulation should significantly enhance the utility of the vaccine in the control of a PPRV outbreak.

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1. Introduction

Peste des Petits Ruminants (PPR) is an acute, highly contagious and fatal disease of sheep and goats and is considered as one of the major constraints to the productivity of small ruminants in African and Asian countries [1]. The causative agent, PPR virus (PPRV), is a member of the genus Morbillivirus within the *Paramyxoviridae* family and is antigenically closely related to the Rinderpest (RP) virus [2], another member of the genus Morbillivirus that causes similar diseases in wild and domestic ruminants. Molecular epidemiology of PPRV based on the 'F' gene sequence from all over the world has defined the existence of four different lineages of virus (I–IV). Of the four known lineages of the PPR virus, lineage I and II have been found exclusively in West Africa, lineage III has been found in East Africa and lineage IV is prevalent in India [3].

For the control of PPR, some attenuated live cell culture vaccines are available. The first vaccine was developed by attenuating the PPRV Nigeria 75/1 isolate by Diallo et al. [4], which belongs to lineage I and is being used for protecting sheep and goats in African countries. Another vaccine, Sungri 96, was developed by attenuating the Sungri isolate of PPRV [5], a virus belonging to lineage IV (Asian lineage) as per F gene sequence analysis [3].

As with all members of the *Paramyxoviridae* family, PPRV is heat sensitive; this is a serious drawback for the efficient use of the live attenuated vaccine in the endemic areas, which have hot climatic environments. In addition, these regions usually have poor infrastructures, being difficult to sustain a cold chain to ensure the maintenance of vaccine potency. Lyophilization in the presence of suitable excipients is a prevailing approach to stabilize such type of biological products. Previous studies with the lyophilized PPRV Nigeria 75/1 vaccine with lactalbumin hydrolysate-sucrose (LS), Weybridge medium (WBM) and lactalbumin hydrolysate-manitol (LM) have shown that the WBM formulation could maintain the virus titer for longer [6]. Different stabilizers i.e., LS, WBM, buffered gelatin-sorbitol (BUGS) and trehalose dihydrate (TD) were also used to prepare the PPRV Sungri 96 vaccine. The results showed that LS and TD allowed for higher stability of the lyophilized PPRV vaccine [7]. LS stabilizer could also maintain the protective titer of the Vero cell adopted Rinderpest vaccine up to 4 hours at room temperature if reconstituted with 0.85% sodium chloride and 1M magnesium sulphate [8]. Another approach to stabilize the PPRV Nigeria 75/1

vaccine was the use of the dehydration method *Xerovac* in the presence of a formulation containing trehalose [9]. Under these conditions the vaccine is stable at 45ºC for 14 days with minimal loss of potency. The World Organization for Animal Health (O.I.E.) recommends the use of the WBM as stabilizing solution for the lyophilized PPRV vaccine. However this vaccine formulation is still very susceptible to thermal degradation [7].

Besides the stability during viral vaccines storage, the process of production can also affect the final virus potency, as the stability of enveloped viruses can be compromised in cell culture bulks due to temperature. Previous studies from our laboratory have shown significant increase in the intrinsic retrovirus stability, also an enveloped virus as PPRV, when production was carried out at high concentrations of glucose or fructose [10]. It was also observed that, independently of the osmotic agent used, higher medium osmolalities enhanced retrovirus stability [11]. Concerning the PPRV stability during production our group also showed that when the virus is produced in a serum-free medium, Ex-Cell™, containing high glucose concentrations, the titers could be maintained for longer than when MEM medium low in glucose was used [12].

In this work, we studied for the first time the effect of the Tris based formulation with trehalose or sucrose on the preservation of the PPRV Nigeria 75/1 vaccine comparing it with the Weybridge formulation actually used for the production of this vaccine [13]. The stability during storage at both refrigerated and high relevant storage temperatures, as well their ability for protection upon lyophilization was evaluated. The effect of medium osmolality on the production and stability of the PPRV was also assessed.

2. Materials and methods

2.1. Cells and virus

Vero cells (ECACC, 84113001) were maintained 37ºC in a humidified atmosphere of 5% CO_2 in air with Minimum Essential Medium (MEM), supplemented with 2 mM glutamine and with 10% (v/v) Foetal Bovine Serum (FBS). All media and supplements were from Gibco. The PPR vaccine strain used was PPRV Nigeria 75/1, kindly provided by Dr. Geneviève Libeau (CIRAD-EMVT,

France), at passage 75. This strain was routinely amplified in Vero cells and stored at -80ºC, using standard techniques [13].

2.2. PPRV production and formulation

Vero cells were seeded at 0.1 x 10^6 cells/mL in 175 cm^2 T-flasks, to a final culture volume of 35 mL per flask. 24-hours (h) after inoculation the cells were infected with PPRV using a multiplicity of infection (MOI) of 0.01. The flasks were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in air. When the cells attained 90-100% confluence the medium was replaced with MEM medium with 2% (v/v) FBS. The cultures were checked daily for development of cytopathic effect (cpe) and the viruses were harvested when the cpe attained 70-80%. All the cells and medium were harvested, freeze-thawed twice to release the cell associated virus and the virus suspension was clarified by centrifugation at 1500 *g* for 10 minutes at 4ºC before formulation. Equal volumes of the virus suspension and different formulations were mixed (See **Table 5. 1** for formulations composition). The lactalbumin hydrolysate, sodium glutamate and Tris-HCl were purchased from Sigma; sucrose, anhydrous trehalose, EDTA and Tween 80 were purchased from Merck; the Hanks' balance salt solution was purchased from Gibco. The formulations based on the Tris buffer were chosen based on formulations used for different enveloped viruses, namely retroviruses [14].

Table 5. 1:Formulation composition.

Formulation	Composition
1 - Weybridge medium (WBM)	2.5% (w/v) lactalbumin hydrolysate, 5% (w/v) Sucrose, 1% (w/v) sodium glutamate, in Hanks' balance salt solution pH 7.4 [13]
2 - Tris buffer (Tris)	20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.02% (w/v) Tween 80
3 - Tris with sucrose (Tris/Suc)	20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.02% (w/v) Tween 80, 1 M Sucrose
4 - Tris with trehalose (Tris/Tre)	20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.02% (w/v) Tween 80, 1 M Trehalose

2.3. Lyophilization

All lyophilizations were done in a Christ Freeze-Dryer Epsilon 2-40 (Germany). The lyophilization protocol was adapted to the lyophilizer from the protocol used at CIRAD, the reference laboratory for PPR [13]. Briefly, (1) Freezing, -40 ˚C for 2 h; (2) Primary drying, -40 ˚C for 14 h at 0.060 mBar; (3) Secondary drying, -35 ˚C for 2 h; -30 ˚C for 2 h; -25 ˚C for 2 h; -20 ˚C for 2 h; -15 ˚C for 14 h; -10 ˚C for 2 h; -5 ˚C for 2 h; 0 ˚C for 2 h; 10 ˚C for 2 h; 20 ˚C for 2 h; all at 0.060 mBar; and (4) Final drying, 35 ˚C for 2 h at 0.060 mBar. One milliliter of the formulated PPRV was dispensed in sterile 5 ml capacity glass vials (Wheaton) and partially sealed with vented rubber stoppers. At the end of the lyophilization process, the caps were closed and the samples were stored at different temperatures until reconstitution for titration. Some samples were reconstituted with 1 mL of distilled water and titrated immediately after lyophilization to evaluate the effect of the process on virus stability. The residual moisture content was determined using the HYDRANAL® - moisture test kit (Fluka).

2.4. Experimental design

The experimental design used in this work is represented in **Figure 5. 1**. The details of different experiments are described in the following sections.

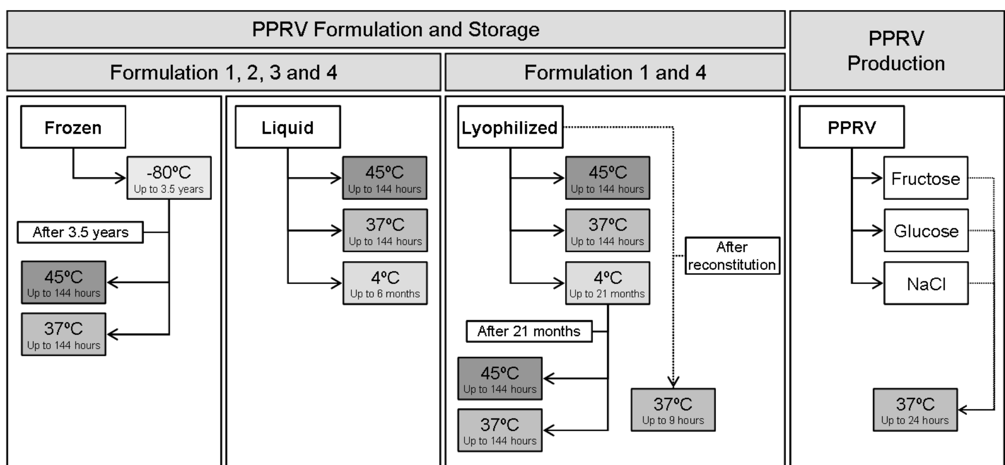

Figure 5. 1: Diagram of experimental design for PPRV storage and production.

2.4.1. Cryopreservation

PPRV liquid samples in different formulations were collected in CryoTubes from Nunc, and the tubes were directly stored at -80ºC; 24 hours after freezing, samples were thawed at room temperature (22-25ºC) and titrated to check the effect of the freezing process in the virus bioactivity. The remaining samples were kept in the freezer to check their stability along time. Studies were performed for a period of up to 3.5 years.

2.4.2. Thermostability testing of liquid and lyophilized PPRV

Sufficient numbers of lyophilized vaccine vials for each stabilizer were stored at 4ºC (up to 6 and 21 months for liquid and lyophilized formulations, respectively), at 37ºC and at 45ºC (up to 144 hours for both temperatures). Samples were taken along time and the lyophilized samples were reconstituted with 1 ml of distilled water. The samples stored at 4ºC were immediately titrated; those samples kept at 37 and 45ºC were stored at -80ºC until titration, in order to perform the titration of the samples at the same time.

2.4.3. Thermostability testing of reconstituted PPRV

Lyophilized vials for each formulation (WBM and Tris/Tre) were reconstituted with four diluents: milliQ water (H_2O), Phosphate-Buffered Saline (PBS), $MgSO_4$ 1 M and Tris/Tre with only 0.5 M trehalose. The osmolality of the suspensions obtained was measured in a Digital Micro Osmometer Type 5R (Hermann Roebling Messtechnik, Germany). For each formulation, three vials were collected and reconstituted separately with 1 ml each of diluent and then stored at 37ºC after dividing into several aliquots in 1.5 ml Eppendorf tubes. Samples were collected every 3 hours for a total of 9 hours and stored at -80ºC until titration, in order to perform the titration of the samples at the same time.

2.4.4. Osmolality effect on PPRV production and stability

To evaluate the effect of the osmolality on viral production, DMEM (Gibco) was supplemented with 10% (v/v) FBS (Gibco), glutamine at 2 mM (Gibco), glucose or fructose (Sigma) at 25, 83 and 139 mM. For sodium chloride (NaCl, Merck, Darmstadt, Germany) supplementation the medium was used with

5.5 mM glucose. Different concentrations of NaCl (12.5, 41.5, 69.5 mM) were added to adjust the medium osmolality to 340, 380 and 430 mOsm/kg. Medium osmolalities were determined by the measurement of the depression of the freezing point using an osmometer (Roebling, Berlin, Germany). The cells were adapted to the different medium conditions after 3-4 passages.

Cells were seeded at 0.1 x 10^6 cells/mL in 25 cm^2 T-flasks, to a final culture volume of 5 mL *per* flask. 24-hours after inoculation the cells were infected with PPRV using a MOI of 0.1. The flasks were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. To monitor the infection kinetics, one flask was harvested daily and its content (cells *plus* supernatant) stored at -80ºC prior to virus titration.

The stability study was initiated by 1:10 dilution of the viral supernatant obtained at 96 hours post infection (hpi) in DMEM without any sugar or osmotic agent. These viral suspensions were incubated at 37ºC in a CO_2 incubator and samples were taken at 0, 2, 4, 8, 12 and 24 hours and stored at -80ºC until the viral titer was determined.

2.5. Virus titration

Virus titration ($TCID_{50}$) was performed with Vero cells in 96 well microtitre plates using standard cell culture procedures [13]. Briefly, 10-fold serial dilution of samples in MEM containing 10% (v/v) FBS were prepared. For each dilution, 12 replicates were done with 100 μL virus suspension and 100 μL cell suspension (0.2x10^6 cells/mL) per well. All the test plates were incubated at 37 ºC in a humidified atmosphere of 5% CO_2 in air. Cytopathic effect was checked on day 12 of titration. End points ($TCID_{50}$/mL) were calculated according to the statistical method of Spearman-Karber [15], simplified for calculations in MS Excel format.

2.6. Data Analysis

Virus stability during storage can be directly correlated with the rate of viral titer decay. Le Doux et al. have shown that the activity of recombinant retroviruses declines at a rate that is proportional to the concentration of infectious virus [16]. In previous work our group has reported this to be the case for retroviral vectors produced by different cell lines [17] and applied also

for retroviral and adenoviral vectors during storage [14]. In such cases, the change in the concentration of infectious viruses with time can be described by equation 1:

$$dX^v/dt = -k_d X^v \qquad (1)$$

which, upon integration, yields the concentration of active virus (X^v) at time t:

$$X^v = X_0^V \exp(-k_d t) \qquad (2)$$

where X^v_0 is the initial concentration of active virus and k_d is the virus decay rate constant.

Although initially developed for retroviral vectors, these equations were applied for PPRV as they also describe well the virus loss of infectivity (see below). To determine the virus decay rate as a function of temperature, PPRV were stored at different temperatures and sampled throughout time. Best-fit values for virus decay rate constants were determined by using non-linear regression analysis to fit the data to equation (2). Vector half-life can be used as a criterion for selecting the appropriate viral formulation. Based on equation (2), the virus half-life, $t_{1/2}$, is given by:

$$t_{1/2} = \ln(1/2) / (-k_d) \qquad (3)$$

Equation (3) is only valid for vector storage in solution at temperatures that do not require freezing (4, 37 and 45ºC). For the storage processes requiring lyophilization and freezing it is necessary to consider the process efficiency, as per equation (4)

$$t_{1/2} = \ln[(1/2)/ \eta_0] / (-k_d) \qquad (4)$$

where η_0, is the storage process efficiency for the virus.

Experimental data for each formulation tested was obtained from triplicate samples taken at specific points in time. Thus, the results presented correspond to means and the errors to the standard deviation. The k_d was estimated through fitting a nonlinear regression to experimental data.

3. RESULTS

The thermolability of live attenuated vaccines is a critical issue for vaccination in developing countries. The main focus of this research was the improvement of the stability of PPRV vaccine. Two strategies were evaluated: (i) firstly, the PPRV stability during storage and (ii) secondly, a strategy to improve the PPRV stability during production.

3.1. Stability during storage

In order to increase the PPRV stability, formulations based on the Tris buffer (Tris), with and without the addition of sucrose (Tris/Suc), or trehalose (Tris/Tre), were tested and compared with the formulation normally used to stabilize the vaccine, the Weybride medium (see Table 1 for formulations composition). The design of the formulations considered: a buffer to avoid pH variations (Tris buffer); the addition of an antioxidant to avoid free-radical oxidation (EDTA); the addition of a surfactant to avoid virus aggregation (Tween 80) and also a stabilizer (sucrose or trehalose) to protect the vaccine during storage at different temperatures. These options were chosen based on formulations used for different enveloped viruses, namely retroviruses [14].

3.1.1. Liquid and frozen formulations

The efficacy of the formulations on the preservation of the titer of the PPRV vaccine was assessed, at first, in liquid and frozen form and afterwards in the lyophilized form. The kinetic profiles and data for virus degradation are presented in **Figure 5. 2** and **Table 5. 2**.

The results showed that PPRV is highly unstable in liquid formulations stored at 37ºC with half-lives ranging from 9 to 21 hours; these values could be increased to days when the temperature was reduced to 4ºC. In the case of the WBM a half-life of 9 hours at 37ºC and of 22 days at 4ºC was obtained. For the Tris/Tre formulation, a half-life of 21 hours at 37ºC and of 30 days at 4ºC could be attained. Tris/Tre and Tris/Suc formulations were also able to retain the PPRV titers above the recommended dose ($10^{2.5}$ $TCID_{50}$/ml) for at least 144 hours at 37ºC, in contrast to the WBM or the Tris formulation which only kept such titers for 72 hours. This data shows that the Tris/Tre formulation presents a superior capability to maintain the PPRV titer under the conditions tested.

At -80ºC the storage process efficacy was very good for all the formulations with an insignificant titer drop after thawing, except for Tris, which presented one log loss after thawing. This was expected since the Tris formulation did not contain a cryoprotector. After the long term storage at -80ºC, the best stability was achieved for PPRV stored in Tris/Suc or Tris/Tre formulations with the titer maintained for at least 3.5 years. It was also observed that at 45ºC all the

formulations lost their titer faster, with more than one log gone in just 3 hours of incubation (data not shown).

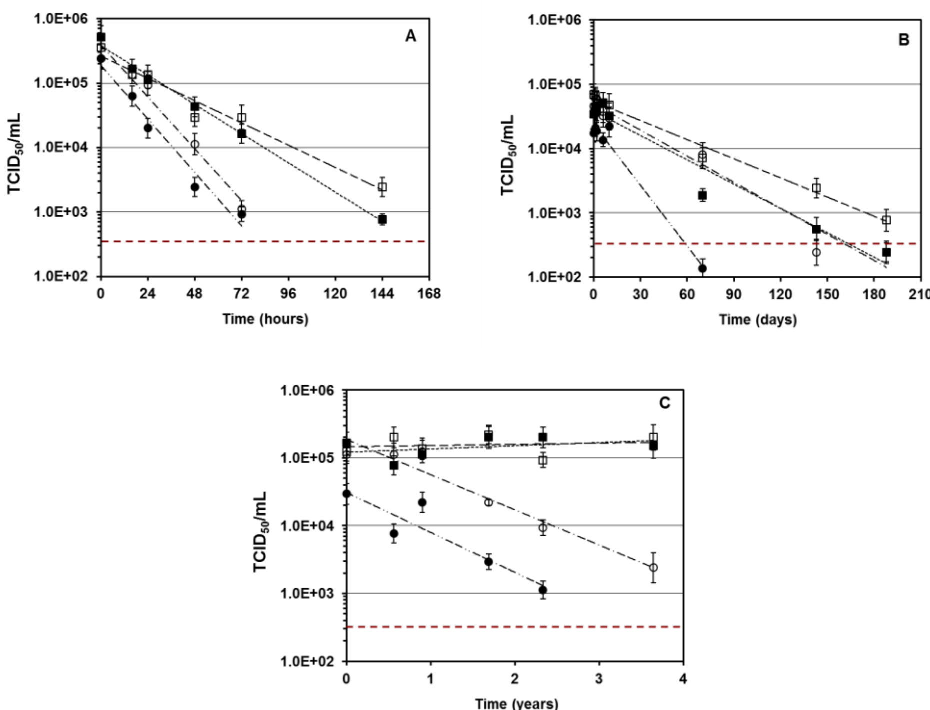

Figure 5. 2: Stability at 37ºC (A), 4ºC (B) and -80ºC (C) for 1:1 dilution of clarified PPR viruses produced in Vero cells with different formulations. WBM (○); Tris (●); Tris/Suc (■); Tris/Tre (□).The different lines represent the linear regressions of virus stability for each formulation tested. The horizontal dashed line represents the value corresponding to the presently used vaccine dose.

Table 5. 2: PPRV decay rate constant (k_d) and half-life ($t_{1/2}$) for each non-lyophilized formulation stored at 37, 4 and -80ºC.

Formulation	37ºC		4ºC		-80ºC	
	k_d (hour^{-1})	$t_{1/2}$ (hour)	k_d(day^{-1})	$t_{1/2}$ (day)	k_d (year^{-1})	$t_{1/2}$ (years)
WBM	0.077 ± 0.008	9	0.031 ± 0.003	22	1.195 ± 0.650	0.6
Tris	0.080 ± 0.009	9	0.073 ± 0.007	10	1.358 ± 0.848	nd[b]
Tris/Suc	0.044 ± 0.002	16	0.029 ± 0.003	24	~0[a]	> 3.5
Tris/Tre	0.033 ± 0.003	21	0.023 ± 0.001	30	~0[a]	> 3.5

[a]No decrease in vector titer was observed for more than 3.5 years; [b]$t_{1/2}$ cannot be determined because the process efficiency is lower than 50%.

3.1.2. Lyophilized formulations

The Tris/Tre formulation described above showed to be a good candidate to protect PPRV during storage and was selected to proceed for the lyophilization study. PPRV with the different formulations (WBM and Tris/Tre) was lyophilized simultaneously using a conventional freeze-drying technique to compare the quality of vaccine after lyophilization. This gave origin to a residual moisture content of approximately 5% for both formulations used, slightly higher than the optimum moisture content recommended by O.I.E for the PPR vaccine, i.e. 3.5%. After lyophilization, both formulations presented a negligible loss of titer, resulting in process efficiencies closely to 100%. The lyophilized formulations were stored at 4, 37 and 45ºC; time decay profiles are presented in **Figure 5. 3** and decay rates presented in **Table 5. 3**. For WBM stored at 45ºC, no virus was detected after 24 hours of storage; at 37ºC its half-life was only 7 hours. In the case of the Tris/Tre formulation a virus half-life of 49 hours and 67 hours, corresponding to only 1.3 logs and 0.7 logs of titer loss after 6 days of storage, respectively, for 37 and 45ºC, was obtained. At 4ºC both formulations showed similar, higher half-lives of 10 months.

Table 5. 3: PPRV decay rate constant (k_d) and half-life ($t_{1/2}$) for each lyophilized formulation stored at 45, 37 and 4ºC.

Formulation	4ºC		37ºC		45ºC	
	k_d (month^{-1})	$t_{1/2}$ (month)	k_d (hour^{-1})	$t_{1/2}$ (hour)	k_d (hour^{-1})	$t_{1/2}$ (hour)
WBM	0.062 ± 0.019	11	0.096 ± 0.020	7	-[a]	-[a]
Tris/Tre	0.069 ± 0.026	10	0.010 ± 0.001	67	0.014 ± 0.006	49

[a]Not determined

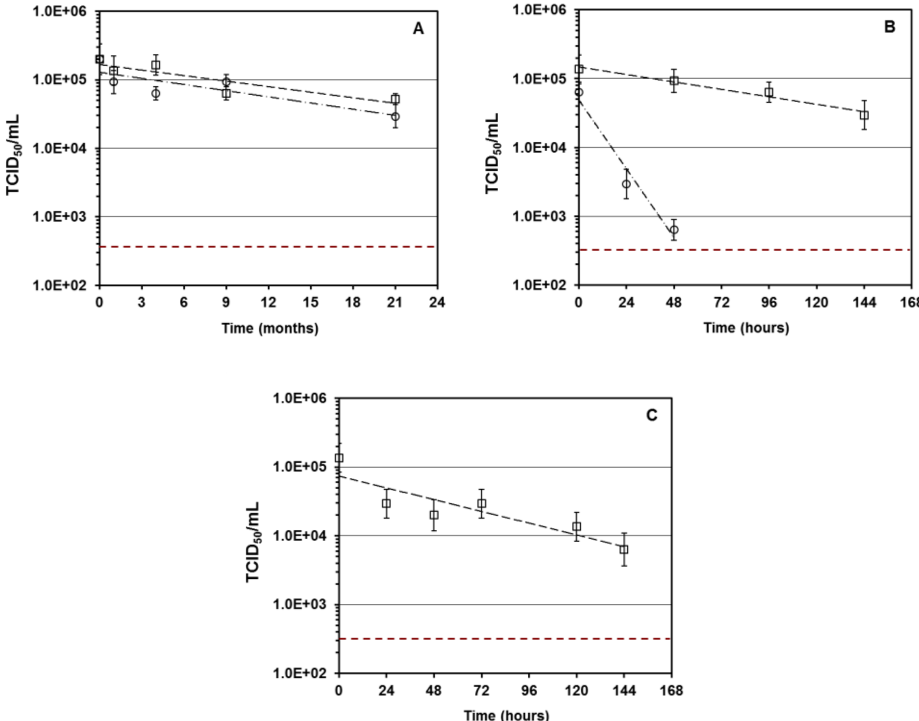

Figure 5. 3: Stability at 4ºC (A), 37ºC (B) and 45ºC (C) of PPRV lyophilized in WBM and Tris/Tre. PPRV were reconstituted using water and stored at -80ºC until titration. WBM (○); Tris/Tre (□).The different lines represent the linear regressions of virus stability for each formulation tested. The horizontal dashed line represents the value corresponding to the presently used vaccine dose. For the WBM at 45ºC no virus was detected after 24 hours of storage.

3.1.2.1. <u>Stability of lyophilized formulations after reconstitution</u>

Currently, the O.I.E. recommends water as the reconstitution solution for PPRV lyophilized vaccine. While water has the advantage of not causing a drop in pH following vaccine reconstitution, it cannot provide any additional buffering capacity of its own; thus different solutions (H_20, PBS, $MgS0_4$ and Tris/Tre formulation with 0.5 M trehalose) were tested for the reconstitution of the lyophilized PPRV for stability at 37ºC for 9 hours. The osmolalities of the suspensions obtained are shown in Table 4 together with the decay rate constants and half-lives. The results showed that for all the solutions tested the virus half-lives at 37ºC varied between 3 and 7 hours. Thus, even with solutions giving rise to high osmolalities (**Table 5. 4**), no impact occurred on PPRV stability.

Table 5. 4: PPRV decay rate constant (k_d), half-life ($t_{1/2}$) and osmolality for lyophilized formulations after reconstitution with different solutions stored at 37ºC.

Solution	WBM			Tris/Tre		
	Osmolality (mOsm/ Kg H_2O)	k_d (hour^{-1})	$t_{1/2}$ (hour)	Osmolality (mOsm/ Kg H_2O)	k_d (hour^{-1})	$t_{1/2}$ (hour)
H_2O	516	0.166 ± 0.026	4	676	0.262 ± 0.083	3
PBS	1062	0.096 ± 0.059	7	1234	0.160 ± 0.084	4
$MgSO_4$ (1M)	766	0.192 ± 0.024	4	928	0.192 ± 0.113	4
Tris/Tre (0.5M)	1496	0.134 ± 0.037	5	1738	0.160 ± 0.084	3

The samples were kept at 37ºC during 9 hours.

3.1.3. Stability after long term storage

The stability of the PPRV at 37ºC and 45 ºC for the frozen formulations thawed after 3.5 years at -80ºC and the lyophilized formulation stored for 21 months at 4ºC was similar to those obtained under the same conditions for the vaccine stored for few days (data not shown). Therefore, either freezing or lyophilization can effectively be used for long term storage of the vaccine.

3.2. Stability during production

The second strategy to improve the PPRV stability was based on culture medium composition manipulation, aiming at improving the virus intrinsic stability. Nowadays PPRV is produced in Vero cells using classical techniques, i.e in T-flasks or roller bottles with MEM or DMEM medium supplemented with low glucose concentrations; the virus obtained has a low stability, as low as 2 to 6 hours half-life at 37ºC [13].

Glucose, along with glutamine, represents the major energetic and carbon sources for most cells in culture. Nevertheless, glucose presents several disadvantages as it is rapidly consumed and inefficiently metabolized, partly being converted to lactate that can be inhibitory to cell growth. Fructose can be used to replace glucose as it originates low amounts of lactate and has been previously shown to enhance retrovirus vector production and stability at high concentrations; irrespective of the osmotic agent used, high medium osmolalities also enhanced retrovirus stability [10, 11].

3.2.1. Effect of increasing osmolality in cell growth and PPRV production

The effect of the cell culture medium osmolality (340, 380 and 430 mOsm/Kg) on Vero cell growth and PPRV production was assessed by the use of increasing concentrations (25, 83 and 139 mM) of the sugar source, glucose or fructose, and the sodium chloride salt (NaCl) (12.5, 41.5 and 69.5 mM) as an osmotic agent. **Table 5. 5** summarizes the results obtained for all conditions tested and **Figure 5. 4** illustrates the Vero cells growth and PPRV production curves using 25 mM glucose (A; control) and 12.5 mM NaCl (B).

Table 5. 5: Effect of increasing osmolality in Vero cell growth and PPRV production.

Osmotic agent	Osmolality (mOsm/ Kg H_2O)	Specific growth rate μ ($hour^{-1}$)	Doubling time (hour)	Max_{cc} [a] (10^6 Cells/ mL)	Max_{icc} [b] (10^6 Cells/ mL)	Max_v [c] (Log_{10} $TCID_{50}$/ mL)
25 mM glucose	340	0.031 ± 0.002	23	2.3	1.8	6.1± 0.2
83 mM glucose	380	0.023 ± 0.004	30	1.7	1.3	6.2 ± 0.2
139 mM glucose	430	0.023 ± 0.001	31	1.1	1.0	6.3 ± 0.2
25 mM fructose	340	0.027 ± 0.001	26	2.2	1.4	7.1 ± 0.1
83 mM fructose	380	0.026 ± 0.004	26	2.0	1.1	6.4 ± 0.1
139 mM fructose	430	0.019 ± 0.004	38	0.7	0.6	5.9 ± 0.1
12.5 mM NaCl	340	0.024 ± 0.003	28	0.8	0.9	6.1 ± 0.3
41.5 mM NaCl	380	0.025 ± 0.005	27	0.6	0.7	5.6 ± 0.3
69.5 mM NaCl	430	0.019 ± 0.003	38	0.4	0.4	4.5 ± 0.4

[a] Max_{cc} – Maximum cell concentration; [b] Max_{icc} – Maximum infected cell concentration; [c] Max_v – Maximum virus titer.

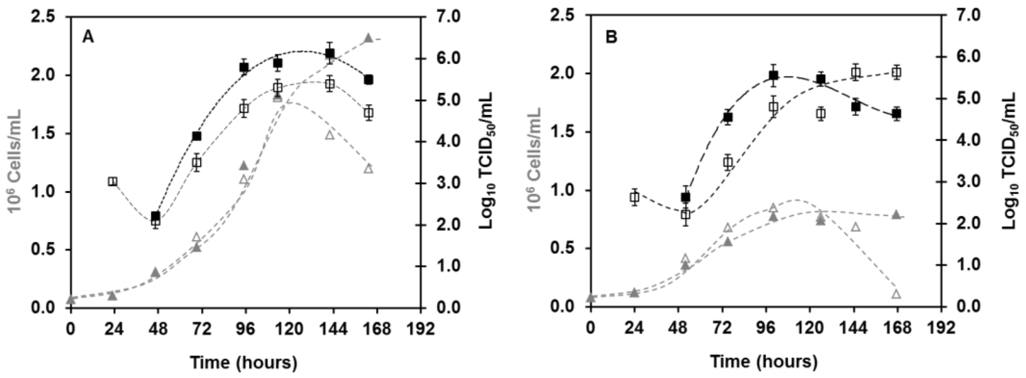

Figure 5. 4: Vero cells growth and PPRV production under different medium compositions.
(A) 25 mM glucose as carbohydrate source; (B) 5.5 mM glucose as carbohydrate source and 12.5 mM NaCl. Non-infected cells (▲); Infected cells (△); Cell associated virus (■); Extracellular virus (□). The lines are guides to follow the progression of the data.

The infection was made at 24 hours post inoculation; since a low MOI is used, only after 48 hours of infection is it possible to observe an increase in the viral titer that follows the growth of the cells. The maximum virus titer is achieved between 72 to 144 hpi and the maximum cell infected concentration around 120 hours of culture. The non-infected cells could grow to higher concentrations than the infected ones when using the carbohydrates as osmotic agents; when NaCl was used the maximum cell concentrations were similar but the viability of the non-infected cells could be maintained for longer. The results showed a decrease in the cellular yields obtained for increased osmolalities. NaCl addition hampered the Vero cell growth when compared with standard 25 mM glucose medium (340 mOsm/kg); at concentrations higher than 12.5 mM the higher osmolalities resulted in lower PPRV titers. The viral yields decreased with the increase of fructose concentration but with glucose the viral titer was kept at higher concentrations. Fructose 25 mM allowed the highest productivity (7.1 $Log_{10}TCID_{50}$/mL), corresponding to 1 log increase in comparison to what happens when glucose is used at the same concentration. During the PPRV production two fractions of the virus are present, an extracellular and a cell-associated fraction. The cell associated fraction is higher than the extracellular fraction by 1 log in virus titer (**Figure 5. 4 A**), when the osmotic agent is a carbohydrate (glucose or fructose). When the NaCl was used at the end of the production phase, there was an inversion of this phenomenon with more virus released to the extracellular fraction as compared to the cell-associated fraction (**Figure 5. 4 B**); i.e. the presence of the salt in the culture medium seems to promote the viral release. To test this hypothesis other experiments were performed. PPRV production was initiated using standard conditions in order to avoid the decrease in the cellular yield and consequent decrease in the maximum viral yield; 72 hpi NaCl was added to the culture medium, 12.5, 41.5 and 69.5 mM concentrations being tested. The amount of viruses present in the cell-associated fraction decreased with the increase of the NaCl concentration and consequently in the osmolality of the culture medium, during the maximum viral productivity phase of production (**Figure 5. 5**), as seen in the previous experiment.

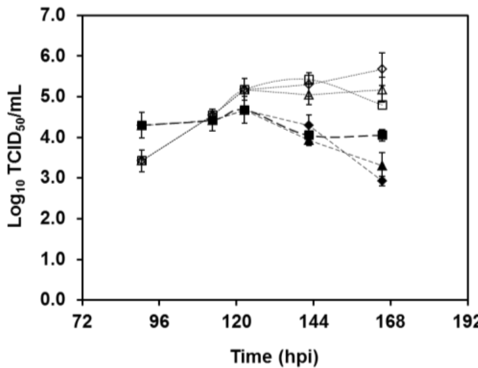

Figure 5. 5: PPRV production in 5.5 mM glucose with addition of NaCl at 72 hours post infection (hpi). NaCl 12.5 mM (■,□); NaCl 41.5 mM (◆,◇); NaCl 69.5 mM (▲,△). Full symbols represent cell associated virus and open symbols represent extracellular virus. The lines are guides to follow the progression of the data.

3.2.2. Effect of increasing osmolality in PPRV stability

Previous studies from our laboratory have identified an important increase in the intrinsic retroviral vector stability when production was carried out at high concentrations of glucose or fructose [10]. Thus, the implication of increased concentrations of the carbohydrate source on PPRV intrinsic stability was studied. The viruses produced under the different conditions were diluted 1:10 in culture medium without the carbohydrate source or NaCl and incubated at 37ºC for 24 hours. The results presented in **Table 5. 6** show that for most of the conditions tested the PPRV stability at 37ºC were in the same range of decay values as those reported in section 3.1.1 for the different liquid formulations.

Table 5. 6: Effect of increasing osmolality on PPRV stability at 37ºC.

Osmotic agent	k_d (hour^{-1})	$t_{1/2}$ (hour)
25 mM glucose	0.125 ± 0.026	6
83 mM glucose	0.067 ± 0.018	10
139 mM glucose	0.057 ± 0.015	12
25 mM fructose	0.043 ± 0.021	16
83 mM fructose	0.109 ± 0.035	6
139 mM fructose	0.088 ± 0.022	8
12.5 mM NaCl	0.099 ± 0.030	7
41.5 mM NaCl	0.145 ± 0.039	5
69.5 mM NaCl	-[a]	-[a]

[a] Not determined

Most of the half-lives are in the 5-8 hours range but when the PPRV viruses were produced using 25 mM fructose the virus half-life increased to 16 hours, whereas with increasing concentrations of glucose (83 and 139 mM) the values were in the 10-12 hours range. This shows slight increases in the viral intrinsic PPRV stability when these culture conditions were applied.

4. Discussion

This study focused on the stability evaluation of PPRV, a heat-labile enveloped virus, during storage and production. Our investigation into the effect of different formulations to protect the PPRV during storage identified a Tris/Tre formulation to yield higher stability, both in liquid and lyophilized form; this formulation originated virus half-lives of 21 and 67 hours at 37ºC, respectively, in the liquid and lyophilized forms; at 4ºC those values increased to 30 days and 10 months, respectively (**Table 5. 2** and **Table 5. 3**). The stability of the PPRV vaccines is normally reported only for lyophilized and not for liquid formulations; thus the liquid conditions evaluated here cannot be directly compared with other data. However our data showed that the Tris/Tre liquid formulation is superior to the WBM, the normally used formulation. This higher stability in liquid gives more flexibility for the preparation of the vaccine solutions prior to lyophilization. At 4ºC, both lyophilized formulations (Tris/Tre and WBM) showed similar, higher half-lives of 10-11 months (**Table 5. 3**). These results are superior to the 30 days reported by Sarkar *et al* for the WBM under the same conditions, for the PPRV Sungri 96 vaccine [7]. At 37ºC, the lyophilized WBM showed an half-life of 7 hours (**Table 5. 3**), similar to the 10 hours reported by Sarkar *et al* for the same formulation used in the PPRV vaccine of the Asian origin [7]. At 45ºC the lyophilized Tris/Tre formulation presented an half-life of 49 hours (**Table 5. 3**) which is similar to the result reported by Worrall *et al* for the *Xerovac* process with another trehalose formulation [9]. Our results at 45ºC showed to be better than those reported for all the formulations tested by Sarkar *et al* yielding half-lives of less than 3 hours at this temperature [7]. Furthermore, the Tris/Tre formulation seems to be less sensitivity to moisture content in the lyophilized form in relation to the WBM, an advantage since often vaccine batches are discarded after lyophilization when

the moisture content is high. In terms of vaccine stability after reconstitution it is important to guarantee that when the vaccine is delivered the correct dose is used. In this work different solutions were tested for reconstitution; for all it was possible to obtain titers above 1×10^4 $TCID_{50}$/mL, used as reference for shelf-life of the vaccine, corresponding to at least 10 vaccine doses in each vial, until the 9 hours of study (**Table 5. 4**). In order to have the higher number of doses per vial after reconstitution, the vaccine should be delivered as soon as possible. Overall, the Tris/Tre formulation presents advantages over the WBM, being a good new candidate formulation for the PPRV vaccine.

Concerning the PPRV production with medium manipulation, in all cases and for all osmolytes (glucose, fructose or NaCl) used, a decrease in the cell yield was observed with the increase of osmolality (**Table 5. 5**), coherent with the decrease in cell growth that has been observed for animal cell cultures grown under prolonged high osmotic pressures [18-21]. An important increment (1 log) in the maximum virus titer obtained was also observed when fructose at 25 mM was used compared to glucose at the same concentration (**Table 5. 5**). For glucose, the titer is maintained with the increase of osmolality; this could be due to an increase in cell-specific productivity, corroborating what is known in different cells namely in hybridoma cells [18], in CHO cells [20, 22] and in insect cells [23], that at higher osmolalites there is an increase in recombinant protein production. On the contrary the increased fructose concentration did not result in an increase of virus productivity; however comparing both sugar sources at the same concentration, productivity is higher when fructose is used except at the high value of 139 mM. This can result from the fact that cells grown with fructose, as the sugar source normally produce lower lactate concentrations due to the slower uptake by the cells, and consequently the pH of the culture is not so affected maintaining the cells in a more physiological environment [24, 25]. It is also known that Vero cells can grow with fructose at concentrations as low as 5 mM [26]; we have tested 10 mM (data not shown) and the results were similar to those using 25 mM, i.e., for these concentrations of fructose the optimum for virus production has been reached. In this case it seems that Vero cells can metabolize well the carbohydrate, in contrast to what happens with other cell types where, the faster metabolization of fructose only happens at higher concentrations [10]. The improvements observed in the maximum titers could not be explained only by the cell culture environment. Therefore other

parameters need to be considered in virus production. It was observed that when the virus was produced at higher concentrations of glucose (83 and 139 mM) or fructose at 25 mM (**Table 5. 6**) the stability of the virus produced increased slightly. The increments verified in the virus stability were not due to chemical protective effects per se: (i) because the infectivity kinetics assays were performed in diluted medium without osmotic agents to ensure that the intrinsic virus stability was being measured and (ii) because in the range of sugar concentrations used normally, the sugar does not show a chemical stabilizing influence [10]. Taking into account that a fraction of the carbon sources in the medium is used to synthesize lipids for the membranes of the producer cells, a correlation between the carbon sources and virus productivity and stability at this level is expected. In the case of glucose, the increase in the virus stability could be due to the fact that higher concentrations increased the osmotic pressure resulting in the rearrangement of the cytoplasmatic membrane composition and increased fluidity, as a result of cell adaptation to the osmotic stress. For retrovirus it has been reported that a decrease in the cholesterol phospholipids ratio due to higher osmolality increased the virus stability [11] and in the retrovirus producer cells glucose plays a major role in lipid biosynthesis [27]. On the contrary for fructose the increase in virus stability was only observed for the lower concentration tested (25 mM) and osmolality increase showed no effect (Table 6). The enhancement in virus stability with 25 mM fructose was superior to those obtained by increasing glucose concentration. This could be due to fructose reducing the lactate production and with that sustain better the pH of the culture, a parameter that can affect the virus stability. On the other hand, it can have an impact on the lipid composition of the cell and virus membrane, independently of the osmotic pressure, probably by being a better lipogenic substrate than glucose as reported by Delhotal-Landes *et al* for human fibroblast cultures [28]. In the case of retrovirus producing cells, the impact of the sugar source in the lipids biosynthesis was more significant for glucose probably because only at higher concentrations does fructose lead to a change in the cell metabolism [27]. Further insights on the metabolism of different sugars and their effect on cell and virus membrane and more detailed studies would need to be performed, namely using a combination of NMR and mass spectroscopy like performed for retrovirus and their producing cells [11, 27]. Concerning the use of NaCl as an

osmotic agent its effect did not result in higher virus productivities mainly because its use reduced significantly the cell growth (**Table 5. 5**). Nevertheless it is worthwhile to highlight that the increase in the concentration of NaCl in the culture medium from the beginning of the culture or after 72 hpi improved virus release, reducing the cell-associated fraction of the virus produced with the highest results obtained at 41.5 mM (**Figure 5. 5**). These results are in agreement with what has been reported for Dengue virus, i.e. increasing osmolalities improves virus production reflected in an increase in extracellular viruses versus cell-associated viruses [29]. The increase of NaCl concentration in the culture medium has also been reported to be important in the release and maturation of some alphaviruses, namely the Sindbis virus, in different cell lines [30, 31]. Moreover this harvesting strategy is scalable and more suitable for a larger scale production than the freeze/thaw cycles normally used. Higher release of the virus from Vero cells is an advantage for PPRV production since it is well known that numerous PPR viruses stay cell-associated.

5. Conclusions

The work presented herein show that an alternative Tris trehalose formulation is superior to the Weybridge medium, recommended by the O.I.E., for PPRV formulation and storage, maintaining the PPRV titers for longer both at liquid and lyophilized forms. This candidate formulation could not only facilitate the transport and preservation of the vaccines, but also all the logistics steps before and after lyophilization, since it presented higher stability in the liquid form and seems to be less sensitive to the final moisture content. These facts could give rise to more batches passing all the quality controls at the end of the lyophilization process, lowering the costs of the vaccine, even taking into account that the candidate formulation could be slightly more expensive due to the higher cost of trehalose in relation to the other components. Regarding the medium manipulation, the use of fructose (25 mM) as a carbon source resulted in 1 log increase in virus yield with a 2.6 fold increase in virus stability compared to glucose. The addition of NaCl to the culture medium promoted higher release of the virus from the cells, ensuring a better harvesting strategy than the freeze/thawing steps. A combination of these production and harvesting strategies with the new candidate formulation could have a major

impact in the manufacturing costs of the PPRV vaccine since higher virus titers with higher stability can be achieved.

The new formulation and the alterations in the production process needs to be validated and tested in animals to confirm the efficacy of the vaccine; nevertheless the presented results constitute valuable information to improve the PPRV vaccine production and stability substantially.

6. Acknowledgments

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7. Author contribution

Ana Carina Silva elaborated the experimental setup and design, performed the experiments, analyzed the data and wrote the chapter.

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Chapter 6

Technology transfer to Ethiopia: A vaccine formulation research and development study

This chapter was based on the following manuscript:

Testing a new formulation for Peste des Petits Ruminants vaccine in Ethiopia. Vaccine, Silva A.C., Yami M., Libeau G., Carrondo M.J.T. , Alves P.M. (2014), *Vaccine*, 32(24): 2878–81

Abstract

In this paper extended tests on a new candidate formulation for Peste des Petits Ruminants (PPR) vaccine carried out at National Veterinary Institute (NVI) in Ethiopia are presented. This work was performed in the frame of the VACNADA project from GALVMED which aimed at procuring vaccines against neglected veterinary diseases to African vaccine producing laboratories, in particular PPR.

After the eradication of Rinderpest, Peste des Petits Ruminants became the next veterinary disease on target for elimination, requiring an effective and thermostable vaccine. In this work, a Tris/Trehalose formulation was evaluated in thermal stability studies, in comparison to the current used formulation of the live attenuated PPR vaccine, the Weybridge medium. The extended results presented herein show an increased thermal stability of the vaccine, especially at 37 and 45ºC, as expected from previously published results (Silva A.C. *et al* 2011). Furthermore, during the course of this project, the NVI teams have clearly demonstrated ability to produce higher quality PPR vaccines after a successful transfer of the technology. These results should significantly enhance the utility of the vaccine in the eradication of PPR.

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1. Introduction

Peste des Petits Ruminants (PPR) is a highly contagious and fatal viral disease affecting sheeps, goats and wild ruminants. The disease is considered as one of the major constraints to the productivity of small ruminants in African and Asian countries [1]. For the control of PPR live attenuated cell culture vaccines based on two strains, PPRV Nigeria 75-1 [2] and PPRV-Sungri 96 [3], are available. The vaccines have been used worldwide with high efficacy in sheep and goat populations, providing at least 3 years immune protection after single dose immunization [4]. The Nigeria 75/1 vaccine strain has proven its effectiveness, regardless of the lineage type circulating in a particular country/region [4].

PPR virus (PPRV) is a member of the genus *Morbillivirus* within the *Paramyxoviridae* family. PPRV is heat sensitive, a serious drawback for the efficient use of the live attenuated vaccine in the endemic areas, which have hot climatic environments. Although infrastructures in these regions are improving, cold chains required to ensure the maintenance of vaccine potency are still fragile. Thus, improvement of the production and stability for the conventional PPR vaccines are worthwhile goals.

Ethiopia has the most important livestock population in Africa, ranked 9th in the world [5]. Sheep and goat populations are estimated to be 20.7 million and 16.4 million respectively, [6] with over 60% of the small ruminants in risk of contracting PPR [5]. The National Veterinary Institute (NVI) is the producing laboratory of the PPR vaccines in the country and also exports the vaccine for neighboring countries. Improving the production and stability of the PPR vaccines produced at NVI will contribute to the control of PPR. This paper presents results from the transfer operation of a new candidate formulation to the production platform of PPR vaccine at NVI and shows the first stability results obtained from this first production batches using the candidate formulation.

2. PPR in the VACNADA project

The programme Vaccines for Control of Neglected Animal Diseases in Africa (VACNADA) aimed to improve the capacity of selected African vaccine production laboratories to make quality vaccines in the quantities required impact on four prioritized neglected diseases of cattle, sheeps and goats, and poultry: contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), PPR and Newcastle disease [7]. This was a 2-year project that finished in December 2011 [7]. VACNADA was implemented by the African Union - Interafrican Bureau for Animal Resources (AU-IBAR) in partnership with the Global Alliance for Livestock Veterinary Medicines (GALVmed), AU-PANVAC and the French Centre for International Cooperation in Agronomic Research for Development (CIRAD).

CIRAD's mission is to contribute to rural progress in developing countries by undertaking research and experiments with partners in these countries. CIRAD operates as a reference laboratory for the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) for animal diseases, notably PPR. It has contributed to scientific knowledge on PPR diagnostic and control. Additionally has developed the live attenuated vaccine against this disease (PPRV Nigeria 75-1) [2]. Within the VACNADA project, CIRAD had supported the activities regarding to PPR vaccine production and control. The work plan to attain the VACNADA project objectives and purposes for PPR was based on the output of an "European Commission" funded project entitled – "Development of marker vaccines, companion diagnostic tests and improvement of epidemiological knowledge to facilitate control of Rinderpest and PPR viruses (MARKVAC)". In this project, the task of optimizing the development of simple and robust methods for the production of the PPR vaccine plus the formulation to enhance stability during storage were accomplished by the Instituto de Biologia Experimental e Tecnológica (IBET). IBET possesses an expertise in the domain of animal cell technology processes for biomedical applications. The proven development results obtained have been previously published in this journal [8, 9]. Work done in the MARKVAC project identified an alternative Tris/Trehalose formulation as being superior to the Weybridge medium, recommended by the OIE, for PPR vaccine formulation and storage, maintaining

the PPRV titers for longer, both at liquid and lyophilized forms. This candidate formulation was transferred to NVI PPR vaccine production platform.

3. Implementation and testing of developed formulation to improve PPR vaccine stability at NVI

3.1. Setting-up the transfer and testing protocol

Detailed information about the production and formulation strategies and the technology available at IBET were transferred to NVI. A work plan for implementation was also set in place. This was followed by a working visit from IBET to NVI for site training, technical discussion and approval of the testing programme, in order to improve the production and stability of PPR vaccine. As planned, NVI had available a vaccine lot at the time, so that the actual tests could be started during this working visit.

During the visit several members of NVI from the production staff to the quality control were involved. Furthermore, members of AU-PANVAC, an independent quality control of the vaccines produced in Africa and concerning PPR, were also involved in the discussion given their experience in the process of thermostability for the Rinderpest and PPR vaccines (Xerovac) [10].

The final programme designed to evaluate the PPR vaccine stability at different relevant temperatures is presented in **Figure 6. 1**, both in liquid and lyophilized form, with the standard (Weybridge medium – WBM) and the new candidate (Tris/Trehalose) formulations as depicted in **Table 6. 1**.

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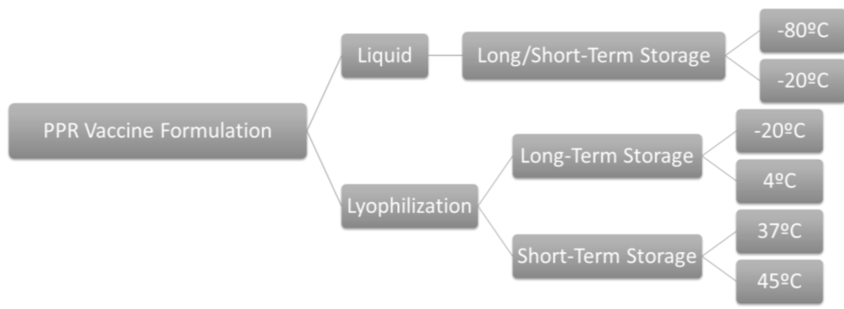

Figure 6. 1: Storage options in the stability studies of PPRV.

Table 6. 1: Formulation composition.

Formulation	Composition
WBM	2.5% lactalbumin hydrolysate (w/v), 5% saccharose (w/v), 1% sodium glutamate (w/v), in Hank's balance salt solution pH 7.2. (use as control, [11])
Tris/Tre	20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.02% Tween 80; trehalose 1 M. [8]

Note: Vaccine liquid bulk was diluted 1:1 with the corresponding formulation.

3.2. Results obtained at NVI

Using the standard Vero cell culture production process in Roux bottles at NVI, two batches were prepared using two different culture media (GMEM vs DMEM), differing mainly in the buffer capacity and amino acid composition, in order to evaluate the production yields. IBET was able to follow the harvesting, formulation, vialling, lyophilization, labeling and first titration of samples before storage and lyophilization at NVI. The competence, operational skills and coordination of the NVI staff in accomplishing all these tasks were outstandingly robust under difficult laboratorial and plant conditions. It became clearly obvious that the technology transfer had been totally successful given the experienced team at NVI.

The first conditions evaluated were the storage of the vaccine bulk with the formulation at -20ºC and -80ºC (**Figure 6. 2**). These conditions are relevant for the storage of the vaccine bulk hold up time prior to quality control release for lyophilization. The results obtained (**Figure 6. 2**) confirmed that it is important to formulate the vaccine before storage to keep the titers as high as obtained during production. Since NVI has a very high track record of production batches passing the quality control tests, the advantage of keeping higher titers during the storage, prior to quality control release for lyophilization, far outweigh formulation extra costs in the few vaccine rejection cases. The results in **Figure 6. 2** indicate that storage in liquid formulation is preferentially better at -80ºC than at -20ºC.

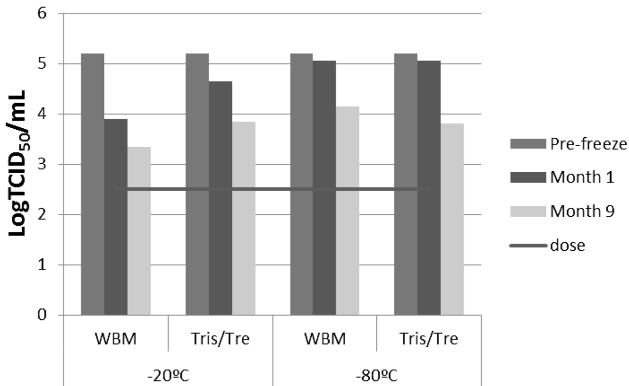

Figure 6. 2: Stability of PPRV in liquid formulations stored at -20 and 80 ºC. The vaccine liquid bulk was diluted 1:1 with the corresponding formulation and stored at the respective temperature.

PPRV, being a live-attenuated vaccine, is particularly unstable at high temperatures, unless stored as a dry product with low residual moisture content. Lyophilization or freeze-drying is the most commonly used method for drying vaccines and other biopharmaceuticals. This process is also used to stabilize PPR vaccine and was maintained for the new candidate formulation. The effect of storage of the lyophilized PPR vaccine at -20, 4, 37 and 45ºC was evaluated and is depicted in **Figure 6. 3** and **Figure 6. 4**.

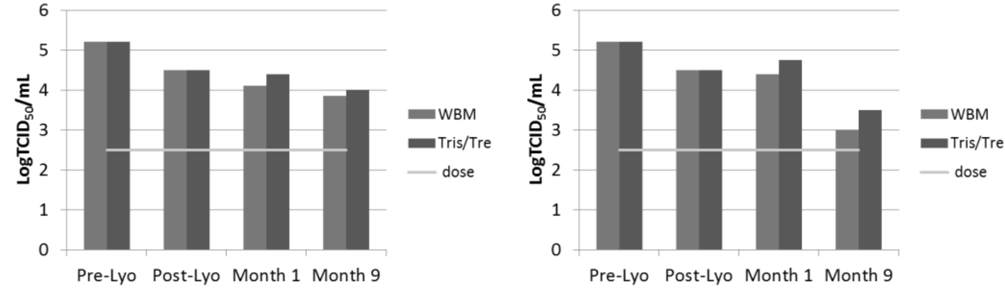

Figure 6. 3: Stability of PPRV in lyophilized formulations stored at -20 and 4 ºC.
The vaccine liquid bulk was diluted 1:1 with the corresponding formulation, lyophilized under standard conditions and stored at the respective temperature.

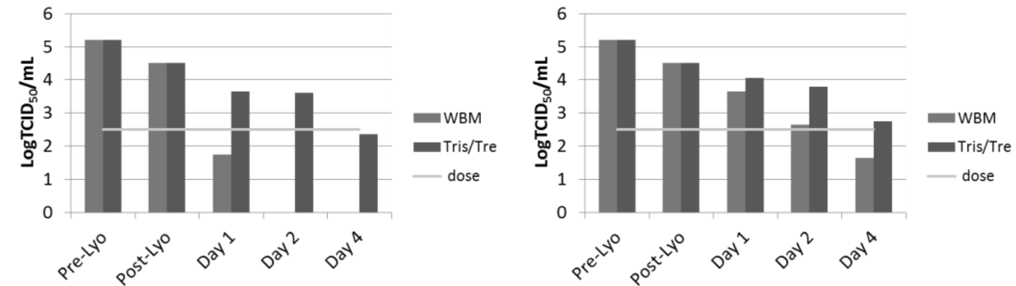

Figure 6. 4: Stability of PPRV in lyophilized formulations stored at 37 and 45 ºC.

The vaccine liquid bulk was diluted 1:1 with the corresponding formulation, lyophilized under standard conditions and stored at the respective temperatures.

As expected, the lyophilized PPR vaccine is even more stable at lower temperatures, with a slightly better stability for formulations stored at -20ºC than at 4ºC, during the 9 months of study. The major differences between the formulations tested were observed at 37 and 45ºC. Under these conditions, the Tris/Trehalose formulation showed to be much more powerful in maintaining titers for the lyophilized vaccine than the standard formulation. These short term stability studies showed also that the production of the PPR vaccine using DMEM medium, instead of GMEM, also enhance stability (data not shown). The results presented herein, showing the Tris/Trehalose formulation substantially improving PPR vaccine storage stability, confirm data from a laboratory test already published for this new candidate PPR vaccine formulation, lasting for 21 months at 4ºC [8]. As these results show a parallel evolution to the earlier laboratory tests, one can envisage a shelf life under lyophilized conditions and storage at 4ºC of at least 21 months.

The costs involved reformulating the existing vaccines can be significant, depending on the changes introduced. In addition to the technical development for a novel, thermostable formulation, the cost of preclinical and clinical testing required to support regulatory approval must be taking into account. In this case, the change is only a few chemicals used for production and formulation as the production process is essentially maintained, making it easier to be adopted sooner than those involving major changes. The media components for the new candidate production and formulation are also already used in other types of vaccines, thus there is no need for regulatory approval of such base materials.

4. Conclusions

The presented results herein confirm that replacing the formulation from Weybridge medium to Tris/Trehalose significantly improves the stability of the PPRV vaccine produced at NVI. It is important to highlight that the decay of the titer for the Tris/Trehalose formulation at 45 °C for 4 days, is less than 2 log and therefore the residual titer is above the minimum acceptable titer ($10^{2.5}$ $TCID_{50}$/mL) and still compatible with vaccine efficacy [11].

This will not only benefit the vaccine producer but also the end users of the vaccine by diminishing the vaccine cost, helping to ensure the effectiveness of the vaccine and reducing dependency on the cold chain.

Thus, it is strongly recommended to:

1 – Use DMEM instead of GMEM medium for production;

2 – Formulate with Tris/Trehalose instead of the normally used Weybridge medium;

3 – Formulate immediately after production so that titer losses currently occurring during the pre-release quarantine can be avoided.

5. Acknowledgements

The authors are thankful for the financial support from GALVmed (VACNADA project) and the European Commission (MARKVAC, FP6-2002-INCO-DEV-1). The authors also acknowledge the people involved in this project, Namely, Dr. Gelagay Aylet, Dr. Woinshet Akalu and Dr. Hassen Belay from NVI, Dr. Karim Toukara, Dr. Sanne Charles Bodjo and Dr. Nick Nwankpa from PANVAC and Dr. Olivier Kwaietek from CIRAD. The author Ana Carina Silva also acknowledges the financial support from Fundação para a Ciência e Tecnologia, Portugal (student grant SFRH / BD / 45786 / 2008).

6. Author contribution

Ana Carina Silva designed the experiments, treated the results and wrote this chapter.

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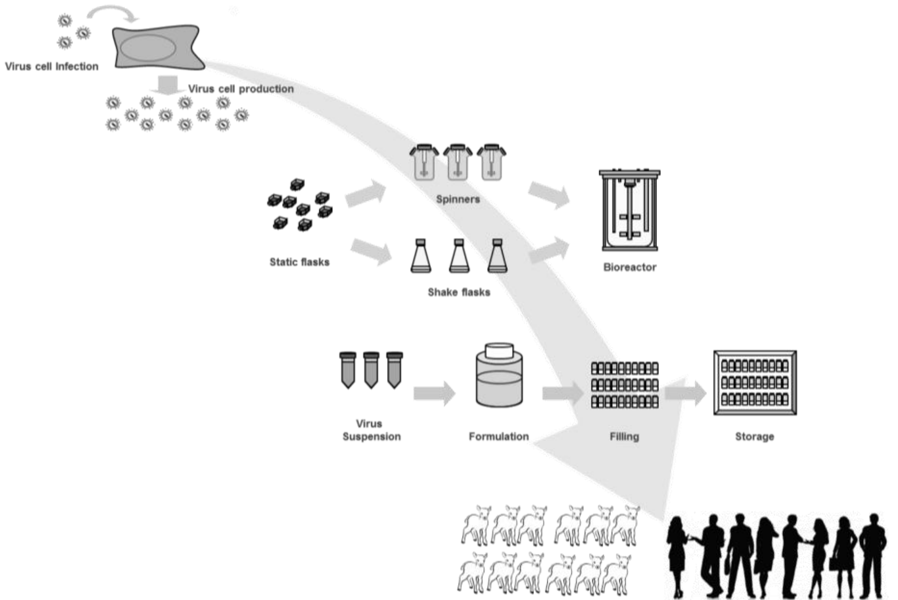
Chapter 7

Discussion and Conclusions



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1. Discussion

Viral vectors are a useful tool to deliver and present vaccine antigens. In the last decades we have witness incredible advances in vaccine development. In fact, the WHO estimates that vaccination has reduced global human mortality rates by infectious diseases by approximately three million deaths *per* year. In addition, the control of livestock health by vaccination increases food security and in developing countries reduces the social economic burden where people sustainability relies mostly on agriculture. Recently, contributed to the eradication of Rinderpest, one of the deadly infectious diseases of cattle [1], was achieved pinpointing the usefulness of committed vaccination plans in improving animal health and ultimately economics.

Viruses are also important players in the gene therapy field. Significant developments occurred with the increasing number of successful clinical trials with promising results and the recent approval of three gene therapy products in the market.

For viral products manufacturing, highly specialized cells are needed, which mainly depend on the desired characteristics of the viral product and the regulatory requirements. Currently, more than 50 cell culture-based human viral vaccines are being manufactured and many more are under development. Given the increasing demand of viral products, the development of robust, scalable and safe cell culture systems for viral production is in the focus of both academic and industrial research. Despite the technological progress and the promising prospects of disease control by vaccination, several challenges still stand.

The work developed in this thesis aimed at overcoming critical bottlenecks in viral production processes. Two distinct viral products, Adenovirus vectors, the most used vector in gene therapy and with high potential also for recombinant candidate vaccines under development and Peste des Petits Ruminant attenuated virus vaccine (PPR vaccine), applied to control PPR, a relevant veterinary disease were investigated. Diverse culture strategies, with different cell lines, were applied to improve quantity and quality of the virus obtained. Both products share similar challenges but there are also product specific bioprocess bottlenecks that need to be addressed. The knowledge

gained with the different strategies used provides novel insights for the implementation of production platforms for different viral products.

1.1. Process development for virus production

The successful production of virus and viral vectors for vaccination relies on robust bioprocesses. The appropriate cell line and optimal process parameters strongly affecting cell growth and virus replication must be addressed (**Figure 7. 1**).

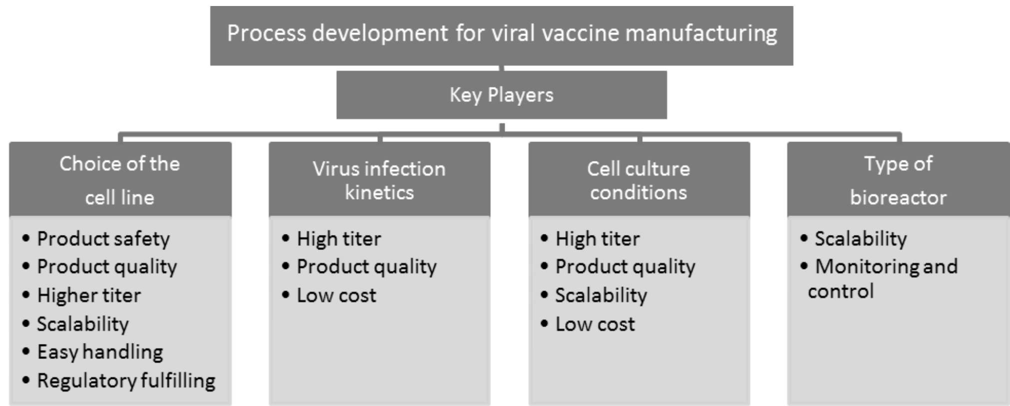

Figure 7. 1: Virus production process requirements.

1.1.1. Choice of the cell line

The ideal production process for a viral or other biotherapeutic product should follow several benchmarks: i) use of cell lines compliant with regulatory safety concerns; ii) use of animal component-free culture medium and iii) rapid production process that yields high concentrations of the product in a cost effective way. The expected virus productivity (cell-specific and volumetric productivities) depends on the virus and the cell line used to propagate it. To address these issues different approaches were evaluated for the two viral products used in this thesis, (i) Adenovirus vectors (**Chapter 2**), and (ii) PPR vaccine (**Chapter 4**).

1.1.1.1. Cell lines for AdV production

Recombinant adenoviral vectors are derived from virus deleted in the E1 gene region; thus, they can replicate only in complementing cell lines that carry the E1 genes. Two recombinant human cell lines are commonly used in the production of such first-generation replication defective adenovirus vectors: a human embryonic kidney cell (HEK293 cells) originally transformed with sheared fragments of adenovirus type 5 (Ad5) DNA [2] and a human embryonic retinoblast cell line (designated PER.C6 cells) transformed with Ad5 E1A and E1B encoding sequences [3]. PER.C6 cells have been preferred for this purpose, since they contain no overlapping sequences with the Ad5 vectors, minimizing or eliminating the probability of generating replication-competent adenovirus (RCA). RCAs are frequently encountered in the large-scale production of recombinant adenovirus vectors with HEK293 cells [4].

In this thesis a human-amniocyte derived single cell clone, 1G3 cells, was evaluated in its ability to produce AdV vectors in a scalable approach (**Chapter 2**). In order to reduce the chances of RCA generation in AdV production 1G3 cells were transformed with a plasmid containing the Ad5 E1A and E1B genes plus modified sequences from the pIX gene, with little overlap between vector and cellular DNA upon vector production. This resulted in no detectable RCA levels in a total of 40 independent small scale assays of 15 serial passages of AdV. On the other hand, in HEK293 cells the presence of RCA was detected in 3 out of 20 assays. These results suggest that 1G3 cells can offer a safer alternative for AdV production, in comparison with HEK293, due to the restricted viral genome sequences that were inserted in the host cell genome.

The 1G3 cell line was successfully adapted grow as single cells in suspension in serum-free medium growing to high concentrations (**Chapter 2**) and producing volumetric AdV productivities in the same range of HEK293 cells. It was identified a safer alternative host cell line for the production of AdV due to the low probability of RCA. 1G3 cells, being a human cell line have the potential to be used for the production of other biologics besides AdV. They have the same cellular origin than CAP cells [5] and those were already evaluated for recombinant proteins production [6, 7] and are capable of producing influenza [8], respiratory syncytial virus and poliovirus [9]. For human use, the produced viruses will replicate in the correct host, glycosylation

of the glycoproteins will have the correct human pattern and highest immune response can be expected [10, 11].

1.1.1.2. Cell lines for PPR production

The PPR vaccine has been manufactured using Vero cells [12]. However the current process of vaccine production still relies in the use of Roux flasks and roller bottles, which present several limitations namely concerning scale-up.

In this work and, since Vero cells used for this process are anchorage dependent, the first attempt to evaluate the production of PPR vaccine in scalable bioreactors was based on the use of microcarrier technology (**Chapter 4**). Vero is currently the most widely accepted cell line by regulatory authorities for vaccine development due to the fact that Vero-derived human vaccines have been in use for nearly 30 years for the production of polio, rabies virus and influenza H5N1 [13], among others [14]. Our results show that PPR vaccine can also be produced in Vero cells using microcarrier technology. The use of serum-free medium not only increased viral yield but allowed its maintenance for a longer period of time (**Chapter 4**). This is a significant improvement since PPR vaccine presents lower stability at elevated temperatures namely at 37ºC, temperature during production. A larger window for harvesting is now possible, facilitating process and leading to higher recovery yields. Starting with higher titers for lyophilization also contributes to improve long thermal stability of a virus preparation during storage as observed for Rinderpest vaccine [15] and Adenovirus vectors [16] where more concentrated preparations presented better stability than those with lower concentration. Nonetheless the increased price of the serum-free medium formulations, the higher yields (vaccine doses per batch) obtained may contribute for the reduction of vaccine production costs.

Recently, Vero cells were adapted to suspension culture [17]. However, the licensing cost for the use of these proprietary suspension cells may be prohibitive for the production of a veterinary vaccine for Africa, as is the case of PPR vaccine. Therefore, alternative cells able to grow in suspension were assessed in their capacity to proliferate the PPR virus (**Chapter 4**). CHO-K1 and MRC-5 cells have proved not to be suitable for PPRV production, eventually due

to the lack of cellular receptors necessary for the virus to infect them [18, 19]. It has been shown that the expression of viral receptors correlates to susceptibility of production of the corresponding virus namely influenza, respiratory syncytial virus and poliovirus in different cell lines [9].

On the contrary, HEK293 and BHK-21A cells were permissive to PPR virus infection, allowing the recovery of similar titers to those obtained in Vero cells using lower MOI's (**Chapter 4**). The lower virus needs for infection with these suspension cells could contribute for the reduction of the master virus bank utilization in the production process. This is very attractive due to the high costs associated to virus bank maintenance. Moreover, in BHK-21A cells higher viral titers are attained earlier in the bioprocess than HEK293 cells, reducing the production process time. The approval by regulatory authorities is facilitated when using cell lines that have already been approved and historically safe in the production of different biologicals. BHK-21A cells are thus a valuable option for PPR vaccine production as they are also the cell substrate for other veterinary vaccines namely Foot and Mouth disease (FMD) [20] for several years. Additionally, the manufacturing facilities can be operated in campaign between PPR and FMD for example, reducing the costs of implementation of a new process.

1.1.2. Cell culture and infection conditions

The viruses used in this thesis belong to distinct families and consequently present completely different life cycles. AdV is a non-enveloped DNA virus with a protein capsid protecting the genome. On the contrary, PPR is an RNA virus with a lipidic envelope that is obtained by budding out of the producing cell. The production process for each type of virus has to be adapted to the virus life cycle to potentiate higher productivities and quality of the desired viral product.

In the case of AdV, a lytic virus, high MOIs are usually used to warranty a synchronous infection. The use of lower MOIs (0.1-0.0001) [21] have been applied for AdV resulting in similar titers than those for high MOIs (5-20). Nevertheless, with low MOIs there is a need of medium replacement every 2 days to maintain the cell growth of non-infected cells which results in higher process times and higher virus volumes to be handled in the downstream processing. The effect of MOI in the production of AdV in 1G3 cells was

evaluated and the results showed that higher titers are obtain using a MOI or 5-10 (**Chapter 2**). The results are in the order of magnitude that is normally used for HEK293 [22, 23] and Per.C6 cells [24].

For PPR production lower MOIs (lower than 1) were assessed (**Chapter 4**). The PPR vaccine was attenuated by serial passage in Vero cells culture and it is known than repeated passage at high MOI can generate defective interfering virus particles (DIP), which can have undesirable measurable biological effects [25-27]. The presence and/or production DIP in higher MOI infections play a role in reducing the production and accumulation of infectious progeny viruses [28]. Krell [29] reported that low MOI minimizes the generation of DIP during successive virus amplification steps and thus increases production of infectious viruses. Based on these observations, the virus master bank used for each production run should be prepared with the lowest MOI possible to ensure reproducibility and higher virus titers in each batch.

We show that for the production of PPR in Vero cells, using microcarriers in stirred conditions (**Chapter 4**) a MOI of 0.01 allows higher infectious titers. For the alternative cell lines permissive to PPR, HEK293 and BHK-21A cells, a lower MOI (0.001) could be applied to originate similar virus titers than those of Vero cells. This approach is an appealing alternative for virus production because an infection strategy based in low MOIs overcome extra virus amplification steps, undesirable in industrial production, and minimizes the virus passage effect. Similar conclusions were reported previously for other virus/host cells systems such as baculovirus/insect cells [30], influenza virus [31], amongst others.

Stirred tank bioreactors (STB) are scalable and hydrodynamically well characterized systems with simple design and operation. In particular, environmentally controlled stirred tank bioreactors allow the on-line monitoring and control of critical culture variables (e.g. temperature, oxygen, pH) known to affect virus productivity (revised in **Chapter 1**).

The capability of using this system for PPR production was evaluated with Vero and BHK-21A cells (**Chapter 4**). The results showed that both cell lines can produce significant titers of PPR ($2x10^6$ $TCID_{50}$/mL) in STB. Nevertheless, upscaling it will be straightforward as BHK-21A cells grow as single cells in suspension. For large scale production of Vero cells inocula preparation is more

challenging since cells must be harvested from microcarrires and transfered to other culture vessels (See **Chapter 1**). For lower scales, up to 5L, the inoculation of the bioreactors can be done with cells from static cultures that are easier to detach from the growing surfaces than those in microcarriers.

1.1.3. Metabolic alterations induced by virus infection

The ''cell-density effect'' in HEK293 and PER.C6® cultures is well described in the literature for AdV production, meaning that cell specific productivity drops with increased of CCIs [24, 32, 33]. This effect seems to limit the range of the CCI to 0.5–1x10^6 cell/mL in order to maintain high productivities. To investigate if the same phenomena occurred for 1G3 cells, the effect of CCI in cells productivity was first evaluated in shake flask (**Chapter 2**). A decrease on the cell specific productivity was also observed for 1G3 cells with increasing CCI's, although at a lower extent then in HEK293 cells. The use of fully controlled STB for AdV production bioprocess resulted in an increase of the productivities for all tested CCIs (**Chapter 2**) in comparison to shake flask cultures. The control of pH in STB may have contributed for the higher productivities than in shake flask which presented, during infection, values lower than 7, which are below the optimum value described in the literature for AdV production (7.2 for HEK293 [34] and 7.3 for PER.C6® [35]). In this system, the decline in cell specific productivities on CCI also occurred for HEK293 with a 10-fold decrease in the specific AdV productivities at the higher CCIs. This effect was completely abolished for 1G3 cells in STB cultures; in fact an increase of 2.9-fold was observed for CCI 3 relatively to CCI 1.

To try to undisclosed why 1G3 cells could produce higher virus titers than HEK293 cells, metabolism of both cell lines was evaluated. From the first analysis (**Chapter 2**), the main carbon sources, glucose and glutamine, were at limiting concentrations at higher CCI's for both cell lines, which could also affect the AdV productivities, as previously reported for HEK293 cells at CCI 3 [22]. 1G3 cells, like HEK293 and PER.C6® cells exhibited a preference for glucose over glutamine metabolism with a ratio $Y_{Glc/Gln}$ in the order of 5–10 mol/mol [24, 36]. Still, in stirred culture systems the $Y_{Glc/Gln}$ was higher for 1G3 cells than for HEK293 cells, which may suggest that glutamine depletion during infection could have less impact on the AdV productivity of this cell line. It has been reported that feeding with glucose and glutamine could not reconstitute

the cell specific productivity at a CCI 3 [22]. In fact, only a complete medium exchange at the time of infection could improve the productivity of HEK293 cells at this cell concentration [22].

The analysis of extracellular metabolites was done by ^{1}H-NMR (**Chapter 3**) aiming to understand the metabolic features of 1G3 cells that could be contributing to the increase productivity at higher CCIs. Metabolic profiling identified significant differences between 1G3 and HEK293 cells in uninfected cultures concerning glutamine and acetate metabolism, as well as different rates of by-product secretion. The main response to adenoviral infection in both cell lines was significantly increased glucose consumption and lactate production rates. Therefore, the stimulation of glycolysis to support production of infectious particles was not cell specific. Performing cultures with and without glutamine confirmed the exhaustion of this amino acid is a main cause of lower adenovirus production at high cell densities, and highlighted different degrees of glutamine dependency of each cell line to meet the energetic and biosynthetic needs for virus replication.

Further information on the intracellular metabolites is needed for a better understanding on the virus-host interactions on the metabolism. A metabolic network for both cell lines could be created and metabolic flux analysis be applied. Based on a detailed understanding of the pros and cons of individual host cells for process performance, properties of other cell lines, specific aspects of virus–host cell interaction and process options can be better evaluated. Overall, a thorough comparison of cell lines might help to develop process optimization strategies, as well as to assess quality differences concerning the virus produced.

1.1.4. Viral stabilization and storage

Although lyophilization technology has resulted in the development of many successful live, attenuated viral and bacterial vaccines, most of these vaccines still require storage at 2-8°C or even below, which necessitates a cold chain for acceptable long term stability [40]. To sustain vaccine efficacy, a cold chain is usually required to maintain vaccine below ambient temperature, from the location of manufacture to the site of vaccine administration. Unfortunately,

cold chain temperature maintenance is not technologically feasible in many remote areas of the world. In fact, because of improper handling techniques, a lack of refrigerators, or power failures, vaccines can be exposed to elevated temperatures for several weeks. Although several guidelines have helped ensure appropriate storage and efficacious vaccine administration, the vaccine cold chain remains error-prone, logistically difficult, expensive, and probably unrealistic in many parts of the developing world [40].

In the case of Adenovirus vectors, the stability issues applied to this virus were not covered during this thesis. In fact several works have been previously done to stabilized this viral product [41-45], including work performed in our laboratory that I also contributed for, using new compatible solutes [46]. We have showed that with a Tris formulation with sucrose or ectoin, AdV could be stored in liquid at 4ºC, presenting and half-life of 87 days. These characteristics facilitate their use as vaccines or viral vectors since no extraordinary stabilization processes are necessary to maintain their efficacy.

PPR vaccine is a safe and efficacious vaccine, however the main constrains of their use is their low stability at higher temperatures by being an attenuated vaccine and an enveloped virus. Two strategies were applied to improve the stability of the PPR vaccine by manipulations: 1) culture medium composition and 2) final formulation (**Chapter 5**). Previous work in our laboratory with retrovirus vectors (also enveloped viruses) have shown that changing the carbon source or osmotic pressure during production could increase the intrinsic stability of the vectors by changing the composition of the lipid membrane [47, 48]. This information was applied to the PPR which resulted also in higher virus intrinsic stability when the production is performed using fructose or higher concentrations of glucose (**Chapter 5**).

To improve the stability of the PPR vaccine during the production process it was also evaluated a strategy for harvesting without the traditional freeze thawing cycles. The addition of NaCl 24h before harvesting allowed the recovery of the cell associated virus without compromising their stability due to the detrimental effects of freezing the virus lacking a freezing protector. This approach has been also efficacious to release dengue virus from Vero cells during production [49]. Moreover this harvesting strategy is scalable and more suitable for a larger scale production than the freeze/thaw cycles normally used.

An alternative formulation (Tris/Trehalose) was recognized has allowing for higher stability than the traditional Weybridge medium under different storage conditions. This formulation could facilitate the production and formulation process before and after lyophilization, since it presented higher stability in liquid form and less sensitivity to final moisture content. More lyophilized batches may pass all the quality controls at the end process, lowering the costs of the vaccine, even taking into account that the candidate formulation could be slightly more expensive due to the higher cost of trehalose in relation to the other components.

Overall, enhancing vaccine stability can help to (1) expand immunization coverage (with minimal increases to the cost of distribution and cold chain upkeep), (2) ensure that administered vaccines are fully potent, (3) reduce vaccine wastage (when high temperature excursions occur or are suspected), and (4) lower inventory turnover (by increasing the product shelf-life of stockpiled vaccines, especially at ambient temperatures). As benefits continue to be demonstrated, immunization programs may increasingly request thermostable vaccine products—further encouraging vaccine manufacturers to meet the commercial demand for them [50].

A better understanding of the molecular basis or the origin of the instabilities of viruses and bacteria should result in the more rational design of stable vaccine formulations. In addition, alternative methods of processing such as spray drying may also lead to improved vaccine stability [51].

1.2. Application of new developments to vaccine production

The Tris/Trehalose formulation for PPR vaccine (**Chapter 5**) was transferred to NVI in Ethiopia under the VACNADA project (**Chapter 6**). This project aimed to support sustainable improvements to the quality and quantity of vaccines of neglected animal diseases namely PPR, in selected African vaccine production laboratories. It was implemented by the partnership between AU-IBAR, CIRAD and GALVmed giving inputs in capacity building through staff development, market intelligence and upgrading of laboratory equipment, facilities and processes.

The technology transfer was successfully accomplished since a good communication between the involved teams was easily conceived. The

competencies, conditions available and needs of NVI were identified and all the information needed was prepared and adapted to the task. A local training was performed with several attendees of the different departments involved in the PPR vaccine production at NVI to guaranty an efficaciously transfer of the technology. After following the processing of the first batch of PPR vaccine with the new formulation (**Chapter 6**) it was possible not only to validate the results obtained in the development (**Chapter 5**) but also to contribute to improve the thermostability of the PPR vaccine produced at NVI placing them in an advantageous position to their direct competitors.

The costs involved reformulating existing vaccines can be significant, depending on the changes introduced. In addition to the technical development for a novel, thermostable formulation, the cost of animal testing required to support regulatory approval must be taking into account. In this case, the change introduced was only in a few chemicals used for production and formulation as the production process is essentially maintained, making it easier to be adopted soon. The media components for the new candidate production and formulation are also already used in other types of vaccines, thus there is no need for regulatory approval of such base materials. Since PPR is a veterinary vaccine, the regulatory restrictions are less stringent than those for human use which facilitate the application of new technologies.

2. Main Conclusions and Future directions

This PhD thesis addressed several challenges that are faced during the development of a virus production process for vaccine or gene therapy applications. This work contributed to the state of the art on process development for viral products in mammalian cells, namely Adenovirus and PPR virus vaccine, but also to other systems that could face similar challenges (**Figure 7. 2**). It covers work in research and development from the selection of the cell line to the final formulation of the biological product and to the transfer of part of the process to a production facility.

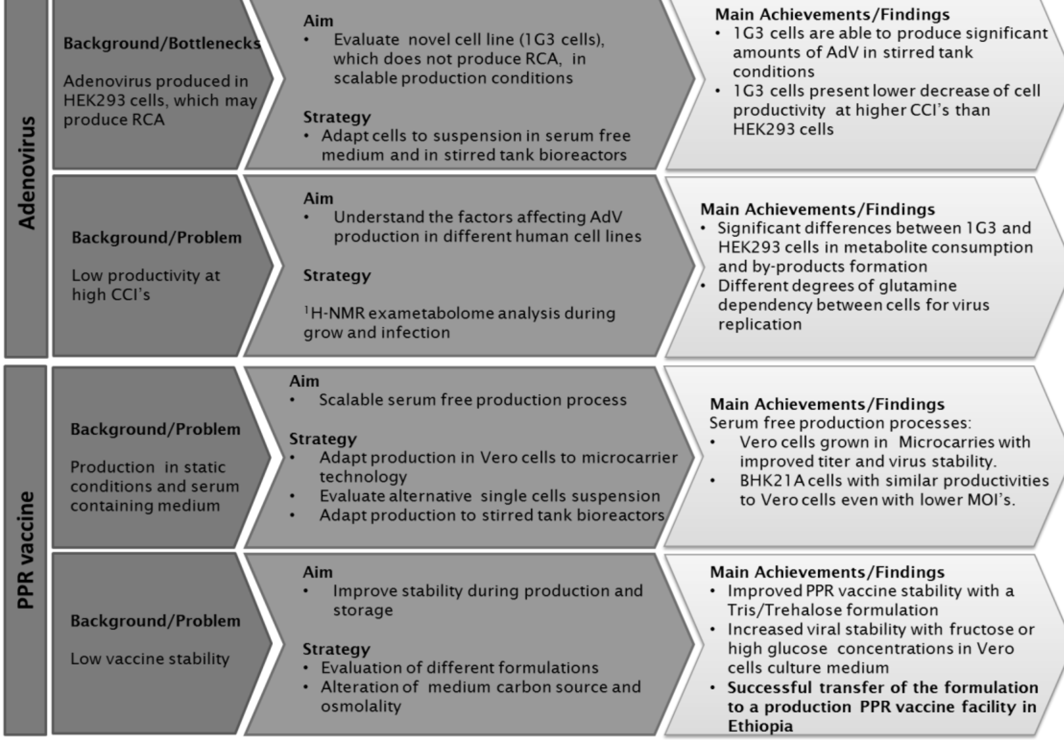

Figure 7. 2: Schematic view of the work developed in thesis including challenges, strategies and outcomes. CCI – cell concentration at infection; MOI – multiplicity of infection; RCA – replicative competent adenovirus.

1G3 cells (**Chapter 2**) are good alternative cell host for the production of Adenovirus vector and PPR virus can be produced in HEK293 and BHK-21A cells (**Chapter 4**). The processes developed in this thesis present better scalability, easy handling, higher titers and product quality (e.g. higher stability) than those traditionally used under the conditions tested (**Table 7.1**).

Table 7. 1: Production processes developed for PPR and Adenovirus production.

Virus	AdV		PPRV			
Process	**Traditional**	**Thesis Output**	**Traditional**	**Thesis Output**		
Cells	HEK293	1G3	Vero	Vero	BHK-21A	HEK293
Medium	Serum-free	Serum-free	Serum	Serum free	Serum free	Serum free
System	Suspension Single cell	Suspension Single cell	Adherent Static	Suspension Microcarriers	Suspension Single cell	Suspension Single cell
CCI/TOI	$1x10^6$ cell/mL	**$3x10^6$ cell/mL**	24h	24h	24h	24h
MOI	5	5	0.01	0.01	0.01-0.001	0.01-0.001
TOH	48h	48h	>96h	>96h	>96h	>144h
Titer	$2x10^9$ ip/mL 1342 ip/cell	**$7x10^9$ ip/mL** **2036 ip/cell**	1×10^6 $TCID_{50}$/mL	**8×10^6 $TCID_{50}$/mL**	**2×10^6 $TCID_{50}$/mL**	1×10^6 $TCID_{50}$/mL

CCI – cell concentration at infection; TOI – time of infection; MOI – multiplicity of infection; TOH – time of harvest; ip – infectious particles; $TCID_{50}$ – tissue culture infectious dose 50%.

Metabolic characterization during growth and Adenovirus production performed by ^{1}H-NMR (**Chapter 3**) showed significant differences between 1G3 and HEK293 cells. Adenovirus replication decreases more drastically at high cell density infection of HEK293 cells than of 1G3 cells.

Concerning PPR vaccine stability (**Chapter 5**), an alternative formulation (Tris/Trehalose) was identified that present's higher performance at elevated temperatures than the one in use (**Table 7.2**).

Table 7. 2: PPRV half-life ($t_{1/2}$) for each formulation stored at 45, 37 and 4ºC.

Formulation		**Traditional**	**Thesis Output**
		Weybridge	Tris/Trehalose
Liquid	**4ºC**	22 days	**30 days**
	37ºC	9 hours	**21 hours**
Lyophilized	**4ºC**	11 months	10 months
	37ºC	7 hours	**67 hours**
	45ºC	- [a]	**49 hours**

[a]Not determined

In the PPR-GEP (PPR Global Control and Eradication Programme) workshop for PPR vaccine producers organized by FAO and OIE (in Kathmandu, Nepal December 2014) several guidelines for the needs of PPR vaccine have been deliberate by all the participants. Most of the laboratories involved in the manufacturing will need to increase their production capabilities to achieve the future vaccine demands and to consider adopting new formulations to provide thermostable products for the eradication campaign to be successful. The results of this thesis contributed to meet those decisions. It was shown not only that using scalable vaccine production processes using Vero or BHK-21A cells could be applied to PPR vaccine (**Chapter 4**), but also a new formulation could improve the vaccine thermostability (**Chapter 5**). The transferred of the Tris/Trehalose formulation to the NVI in Ethiopia (**Chapter 6**) showed that with proper training, good communication and collaboration between experts, the needed alterations in the production laboratories could be successfully accomplished.

Still, to achieve a robust and efficient virus production platform, some trends identified throughout this thesis need verification and other technologies could be applied:

- The use of disposable, single-use bioprocessing technologies, such as bioreactor bags in PPR vaccine and AdV production could be also evaluated since they eliminate cleaning validation steps and provide significant time and cost savings.
- The application of metabolic flux analysis combined with non-invasive and high content analytical techniques like NMR with labelled substrates will allow identified the metabolic requirements for cell growth and viral production that affect productivity.
- The knowledge gained by the identification of the limiting metabolic nodes may be used to improvement the viral productivities and surpass the “cell density effect” by using target metabolic engineering as already obtained for other systems.

3. Author contribution

Ana Carina Silva wrote this chapter.

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