Optimization of Mammalian Reovirus T3D Growth in L929 Cells

By

Abdullah Awadh

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology and Infectious Diseases
University of Manitoba
Winnipeg, Manitoba
CANADA

Abstract

For over half a century, cell culture systems have been contributing to the current elucidation of endless arrays of biological mechanisms. Viruses are not an exception. The recognition that viruses could be isolated and propagated in tissue cultures became possible after extensive improvement and optimization procedures to the initial success of growing animal cells in vitro. This achievement has resulted in protecting mankind from devastating infectious diseases, including complete eradication and significant reduction in morbidity and mortality through vaccines and antiviral agents and promising trials in developing solutions against metabolic disorders and cancers through gene therapy and oncolytic agents.

Experimental and clinical trials have been increasingly showing that mammalian orthoreoviruses are attractive candidates as natural oncolytic agents against a wide range of solid and hematological cancers. Advancement of such trials requires efficient productive systems capable of generating enormous amounts of oncolytic reoviruses at reasonable cost. Taking into account this demand for high scale production of mammalian orthoreoviruses (T3D in particular), very few reported studies have tried to understand the factors that affect its production and subsequently optimize them to maximize the virus yield in cell culture systems.

For this study, I manipulated several growth parameters (multiplicity of infection (MOI), cell density, cell feeding, media pH, and flask size) sequentially to select optimal conditions. Manipulation of cell density, whether cells were fed or not, and flask size all led to moderate changes in progeny virus titer. Altered media pH led to dramatic (more

than 100-fold) virus replication changes. We conclude that cell physiological status has a key impact on infection progression and virus production. Optimal parameters consisted of initial infection at MOI of 0.1, cell density at infection of 95%, media re-feeding, and media pH 7.0-8.5. Slightly greater titers were obtained in smaller flasks.

Acknowledgments

With sincere gratefulness, I would like to thank King Saud bin Abdulaziz University for Health Sciences for the scholarship grant to accomplish graduate studies abroad. It was through the Saudi Arabian Cultural Bureau in Canada that maintained this generous grant through uninterrupted coverage to tuitions, health, travel, and living expenses.

I would also like to express my wordless indebtedness to Professor Kevin Coombs for the kind host of my research work in his lab, honoring the opportunity to benefit from his distinguished knowledge and experience, and for the boundless support and invaluable advices throughout the period of the program.

I also wish to extend my gratitude to the department of Medical Microbiology at University of Manitoba for the admission into the program and limitless knowledge and experience acquired from the distinguished professors in the department and researchers in the National Microbiology Lab. In particular, I would like to thank Dr. John Wylie and Dr. Michael Butler who were the members of my research supervision committee.

It is also my acknowledgment to the Manitoba Centre for Proteomic and Systems Biology for the access to the lab facilities and expertise. In addition, I would thank members of Professor Coombs lab (Alicia, Andrea, Anh, Kola, Niaz, Saeedah, and Thais) for sharing assistances, ideas, and friendship.

Dedication

From the bottom of my heart, I dedicate this work mostly to my parents. Unfortunately, my father would not be able to recognize it due to senile dementia, but my beloved mother who has encouraged my departure for study, committed for my father care, and never quit supplication for my welfare. Yet, she could not have stood for that without backing by my great sisters and their husbands who have been always up to my parents needs.

I dedicate it to my wife Lamees who through her patience and charming care, made it possible to overcome the foreignness pain and progress successfully toward the accomplishment. Finally to my newborn daughter Lara who had to be born away from many people craved to share her moment.

Table of Contents

List of Tables

List of Figures

1 Introduction

1.1 Propagation of Viruses

1.1.1 History

At the beginning of the last century, occasional reports of noticeably promising trials of growing viruses in tissue fragments in vitro had started to appear in the biomedical research literature, but it was not until after World War II, that tissue cultures were seriously considered as novel tools for studying viruses. The main reason that hindered the usefulness of this new approach was contamination; development of antibiotics followed by improvement in procedures has helped greatly in solving this problem. It was at this point that virologists recognized that such cultures could be utilized for isolating and propagating viruses (Kuchler 1974).

One of the early investigators to study viruses in tissue cultures was John Enders, who showed –as an early major discovery- that viral tropism was not absolute. Instead, he found that a neurotropic virus like poliovirus (considered neurotropic), was capable of growing in various embryonic tissue cultures in vitro. This work was published in Science in 1949 (Enders, Weller et al. 1949).

Such discovery led to screening and optimization of efficient tissue culture systems capable of producing mass amounts of viruses for vaccine development. One of these was the monkey kidney tissue cultures for poliovirus, which let Jonas Salk prepare the inactivated poliovirus vaccine through attenuation by formaldehyde treatment (Youngner, Ward et al. 1952; Salk, Krech et al. 1954). Additional substantial works were then showed by Albert Sabin and Hiliary Koprowski on the attenuation of poliovirus virulence. The viral strain developed by Sabin was then the official strains licensed for

the commercial production of vaccines in US (Sabin 1965). In 1954, applying comparable procedures, Enders, in association with Thomas Peebles, isolated the virus of measles in human and monkey kidney cell cultures (Enders and Peebles 1954). These techniques offered for the first time a consistent and reliable means of propagating this agent under laboratory conditions (Kuchler 1977).

1.1.2 Usages

The availability of efficient virus propagation protocols is paramount in advancing research in numerous biomedical disciplines. Some of these include studies that aim to provide an insight into the process of virus morphogenesis, characteristics of virus components, molecular biology of cell, pathogenesis and immune response, vaccine research and synthesis, and most recently the application in cancer virotherapy (Faisst 1999; Castilho 2008).

1.1.3 Methods

Before the advent of cell cultures, whole laboratory animals or embryonated hen's eggs were the main experimental tools for propagation and purification of viruses. They are infected through the natural route and the virus is allowed to replicate either locally or systemically for certain period of time. The animals are executed, and the host organs are then subjected to multiple procedures to finally obtain the cell-free extracts which contain the progeny viruses. Fertilized hen eggs can also support virus growth in which most viruses grow in the embryonic membranes (e.g. herpesviruses, poxviruses, influenza virus, and mumps virus). Laboratory animals and embryonated eggs are being replaced by cell culture systems, which are more convenient, easy to handle, and better to control. However, animals are still the appropriate propagation systems for viruses like

arboviruses and rabies virus, and eggs for orthomyxoviruses. In addition, animals are still the model of choice on studies on viral pathogenesis and vaccine production (Faisst 1999).

1.2 Tissue Culture

1.2.1 History

During the early part of the last century, tissue culture was for the first time used to study cell behavior in vitro; under no systemic variation or experimental stress (Carrel 1912). The initial techniques involved monitoring cell migration from fragment of tissue and mitoses in the margins. For more than 50 years, these methods of deriving cells from primary tissue had dominated the field (Fischer 1924), and hence the name “tissue culture” is still in use as a general term although it was the use of dispersed cell cultures throughout the last 60 years that contributed to the unprecedented expansion in this field.

Rous (Rous and Jones 1916) was the first to plate dispersed cells after disaggregation of explanted cells. Cell strains were generated by passaging (surgical subdivision earlier) of the culture. Sanford et al (Sanford, Earle et al. 1948) have isolated – what would be the first cloned cell strain – L929 from mouse L-cells by capillary cloning. In the 1950s, numbers of key milestones were established in the development of tissue culture techniques. These include the use of trypsin for subculturing and generation of single cell suspension which helped in advancing the cloning of single cells, and procedures for viral plaque assays in monolayer cultures by Dulbecco (Dulbecco 1952). Moreover, the first continuous human cell line, HeLa was established by Gey (Gey and Bang 1951). At this stage, maintenance and propagation of cell lines became feasible, largely due to the introduction of antibiotics. Defined media were also developed in

1950s (Morton, Morgan et al. 1950; Parker, Healy et al. 1954; Eagle 1955; Lockart and Eagle 1959; Waymouth 1959), which subsequently led to the development of serum-free media (Ham 1963; Ham 1965).

1.2.2 Principles

The term "cell culture" is defined as in vitro cultures of dispersed cells derived from original tissues by various means; enzymatic, mechanical, or chemical disaggregation (Faisst 1999). Either primary explants or cell suspensions could serve as a source of cell cultures (Butler 2004; Freshney 2005). These cells could be propagated by passaging; the resultant daughter cultures constitute the first step in the formation of a cell line. The cell lines can be characterized and sustain these features throughout their life span. Alternatively, cell lines can be made continuous – mainly by transformation – and subsequently know as a cell strain upon selection (MacDonald 1990; Masters 2000).

The usage of tissue culture systems in biomedical research has many advantages. Some of these include: control of the environment, characterization and homogeneity of sample, economy, scale, mechanization, and in vitro modeling of in vivo conditions. Limitations on the other hand include expertise, quantity, differentiation, selection, origin of cells, and instability (Freshney 2005).

One aspect in cell culture technology might constitute discrepancies in cell behavior between cultured cells and their counterpart in vivo. This obstacle arises from the dissociation of cells from a three-dimensional geometry into a two-dimensional substrate. As a result, some specific cell interactions peculiar to the histology of the tissue are lost. Additionally, in two-dimensional growth, the cells spread, become mobile, or

start to proliferate, therefore increases the growth fraction of the cell population (Masters 2000; Butler 2004; Freshney 2005).

1.2.3 Application

The applications of tissue culture are ever-increasing since their first introduction into the field of biomedical research. The true explosive expansion was after establishment of the principles of genetics and molecular biology. Some of these applications include intracellular activity, intracellular flux, environmental interaction, cell-cell interaction, genetics, and cell products. Studies on these aspects elucidated invaluable information on the mechanisms of numerous biological processes such as replication and transcription of DNA, protein synthesis, energy metabolism, drug metabolism, translocation of hormone, signal transduction, membrane trafficking, nutrition, infection, cytotoxicity, carcinogenesis, morphogenesis, proliferation, adhesion, motility, metabolic cooperation, genome analysis, genetic manipulation, transformation, immortalization, secretion, biotechnology, product harvesting, and downstream processing (reviewed in Rapp and Melnick 1964; Klein-Szanto, Terzaghi et al. 1982; Gaicu 1987; Spier 1991). Furthermore, tissue culture technology is a major part in the development of antiviral vaccines.

1.3 Cancer and Viruses

The ultimate aim of optimizing efficient in vitro system for production of reovirus in this study is to be employed in the therapeutic battle against cancers. Cancer affects millions of people every year and has become a driving force in medical research, responsible for significant amounts of research expenditures and government funding. Progress has been made in all aspects of combating cancer, including improved

techniques in early diagnosis, advances in surgery, improved chemotherapy, hormonal therapy, immunotherapy, gene therapy, and radiation therapy (Davis and Fang 2005). Despite these advances, however, Cancer is still a leading cause of death worldwide; it resulted in 7.9 million deaths in 2007 and is anticipated to continue rising to 12 million deaths in 2030 (WHO). In the United States, cancer is the second leading cause of death, responsible for killing more than 1500 people every day. An estimated 1.5 million new cancers would have been diagnosed and 0.5 million would have died during 2010 in the United States alone, costing more than $263.8 billion in medical expenses and loss of productivity in 2010 (Society 2010). Clearly, new therapies are needed that are capable of treating and preventing the formation of cancers.

1.3.1 Cancer virotherapy

Virotherapy is a recent approach incorporated in cancer therapy. By definition, it is the employment of viruses, whether natural or genetically modified, to selectively target tumor cells for replication and eventually killing (Davis and Fang 2005).

Viruses are designated “Oncolytic” when they are capable of selective replication – which leads to destruction – of tumor mass with minimal collateral damage to normal tissue (Stanford, Bell et al. 2010). Their replication is sustained in neoplastic tissues which are characterized by accelerated proliferation, hence providing regenerated cell population to infect. Theoretically, if these oncolytic viruses (OVs) could sustain their persistence at cellular and systemic levels, it could be speculated that eradication of certain types of metastatic tumors might be possible by single therapy (Davis and Fang 2005).

The principle of viral oncolysis was initially recognized in the beginning of the 20th century. Throughout the 1950s and 1960s, potential oncolytic viruses were evaluated experimentally as well as in humans. The interest in viral oncolysis had fluctuated and raised many controversial issues mainly about the side-effects and the unexpected outcomes soon after the first clinical trial which used a vaccine strain of rabies virus against melanomatosis in 30 patients. The clinical trials were ceased until the concept of gene therapy accompanied by breakthrough in biotechnology had been established near the end of that century. During the last 15 years, a second wave of interest in viral oncolysis has commenced with very powerful tools and technology which has resulted in the consensus notion that virotherapy may be established as one of the approaches of cancer treatment alongside previous ones (Vaha-Koskela, Heikkila et al. 2007).

1.3.2 Reovirus-based cancer therapy

Reovirus is a double-stranded RNA virus which is considered among the most promising oncolytic viral agents which demonstrate oncolytic capability against variety of cancerous cell lines, tumors, and in clinical trials in human. Additionally, reovirus possesses some of the most important features sought for an ideal oncolytic virus. The main one is the exploitation of abnormal cellular signaling usually found in cancerous cells. Others include genomic stability, production easiness, and minimal toxicity to human body. Moreover, reovirus poses no clinical disease manifestation according to the considerable literature related to reovirus infections in adults and the virus is usually isolated from respiratory and gastrointestinal tracts in humans. These features alongside extensive pre-clinical efficacy, affirm the position of reovirus as an attractive candidate to

be seriously considered in further human clinical trials (Thirukkumaran and Morris 2009).

As with any therapeutic approach, there are limitations in the application of oncolytic virotherapy. The most critical ones are the immune response, safety (especially with modified agents), and requirement of local administration. These challenges and others are under intensive studies for solutions capable of minimizing their effects to keep the option sustained (Davis and Fang 2005). But another important constraint is the steady supply of mass amount of oncolytic inoculums of potentially successful candidate has not been sufficiently addressed, or for which very limited publications exist. For a particular oncolytic candidate to gain acceptance and eventual approval, a significant literature body needs to exist to support the concept. Building a supportive literature body requires considerable number of studies and trials to be conducted at wide range to include for example various sites, settings, and patient populations. The progression of such process might be hindered or delayed due to the lack or interruption of sufficient supply of the oncolytic agent under evaluation, hence such work may provoke more interest in expanding research in this area.

1.4 Reovirus Features

1.4.1 History

The reovirus (respiratory enteric orphan virus) designation was proposed by Albert Sabin in 1959 (Sabin 1959) to include a group of respiratory and enteric viruses that cause no human disease and share distinct features. These initial characteristics included: size, cytopathic effect in tissue culture (monkey kidney cells), pathogenicity pattern, and haemagglutination of type O erythrocytes. There are three reovirus serotypes

in which their prototypes were all isolated from stools; type 1 Lang (T1L) by M Ramos-Alvarez and AB Sabin in 1953, type 2 Jones (T2J) by the previous two researchers in 1955, and type 3 Dearing (T3D) by the same two researchers in 1955 and by L Rosen in 1957 (Tyler 1999; Olland, Jane-Valbuena et al. 2001; Tyler, Clarke et al. 2001; Coombs 2006).

1.4.2 Classification

Mammalian orthoreoviruses (MRVs) represent the prototypic members of the genus *Orthoreovirus* in the *Reoviridae* family. There are currently 12 genera in this family. Viruses in this family lack envelope and have intermediate structural complexity. Their genomes are segmented (10–12) double-stranded (ds) RNA surrounded by multiple (2 or 3) protein capsids (Coombs 2006). Other viruses in this family are the rotaviruses, one of the significant viral agents associated with viral gastroenteritis worldwide (Offit 1994; Estes, Kang et al. 2001; Kapikian 2001). Orbiviruses, which are the economically important insect- vectored are another group in this family (Roy 1996). Many of these viruses infect plants, vertebrates, and invertebrates.

1.4.3 Structure

The genome of MRVs has ten segments of dsRNA encapsidated in two shells of capsid proteins composed of eight different proteins (**Figure 1**). It consists of three large segments (L1, L2, L3), three medium segments (M1, M2, M3), and four small segments (S1, S2, S3, S4) with approximate size range of 3.8-3.9 kilo base pairs (kbp), 2.2-2.3 kbp, and 1.2-1.4 kbp respectively. The total genomic size is around 23 kbp (Joklik 1999; Clarke and Tyler 2008).

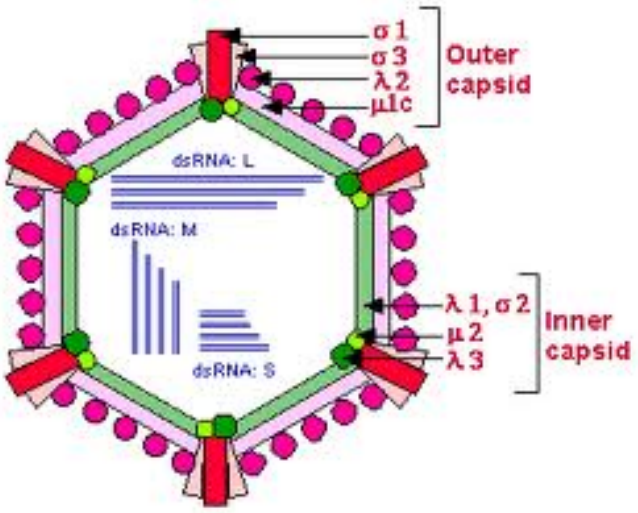


Figure 1. Structure of the reovirus virion. Schematic representation of the reovirus particle showing the 10 dsRNA segments and the major structural components.

Source: http://www.microbiologybytes.com/virology/Reoviruses.html.

The reovirus inner capsid encloses the viral genome composed of five proteins λ1, λ2, λ3, μ2, and σ2. The approximate diameter of the core, which has icosahedral symmetry is, 52 nm and around 58 nm with the spikes. This inner capsid, which is also called “core”, provides the machinery complex for mRNA transcription (Olland, Jane-Valbuena et al. 2001; Liemann, Chandran et al. 2002; Coombs 2006).

The outer viral protein shell (outer capsid) is composed of three proteins. Proteins μ1 and σ3 (600 copies each), and protein σ1 (up to 36 copies). Outer capsid proteins are organized in a fenestrated T=13(l) lattice. Proteins within this layer are responsible for host cell recognition and in the entry of the viral core into the cellular cytosol (Strong, Leone et al. 1991; Dryden, Wang et al. 1993; Coombs 2006).

1.4.4 Replication

Steps involved in the replication of reovirus occur in the cytoplasm with no evidence suggesting significant nuclear involvement (**Figure 2**). The first step is the attachment of the viral particle to the surface of the target cell. This event involves the binding of viral protein σ1 with cellular receptor. Junction adhesion molecule (JAM) is one of the possible receptors (Barton, Forrest et al. 2001). The second step is the virus entry into the cell. There are two possible mechanisms by which reovirus can enter the cell. The first is through receptor-mediated endocytosis, the mechanism describes entry of the intact virion which then undergoes proteolysis and converted to what is known as intermediate or infectious subviral particle (ISVP) (Bass, Bodkin et al. 1990). The second is through direct penetration of the cell membrane, the mechanism used by ISVP which can be generated either naturally (upon exposure to intestinal proteases) or experimentally (Odegard, Chandran et al. 2004).

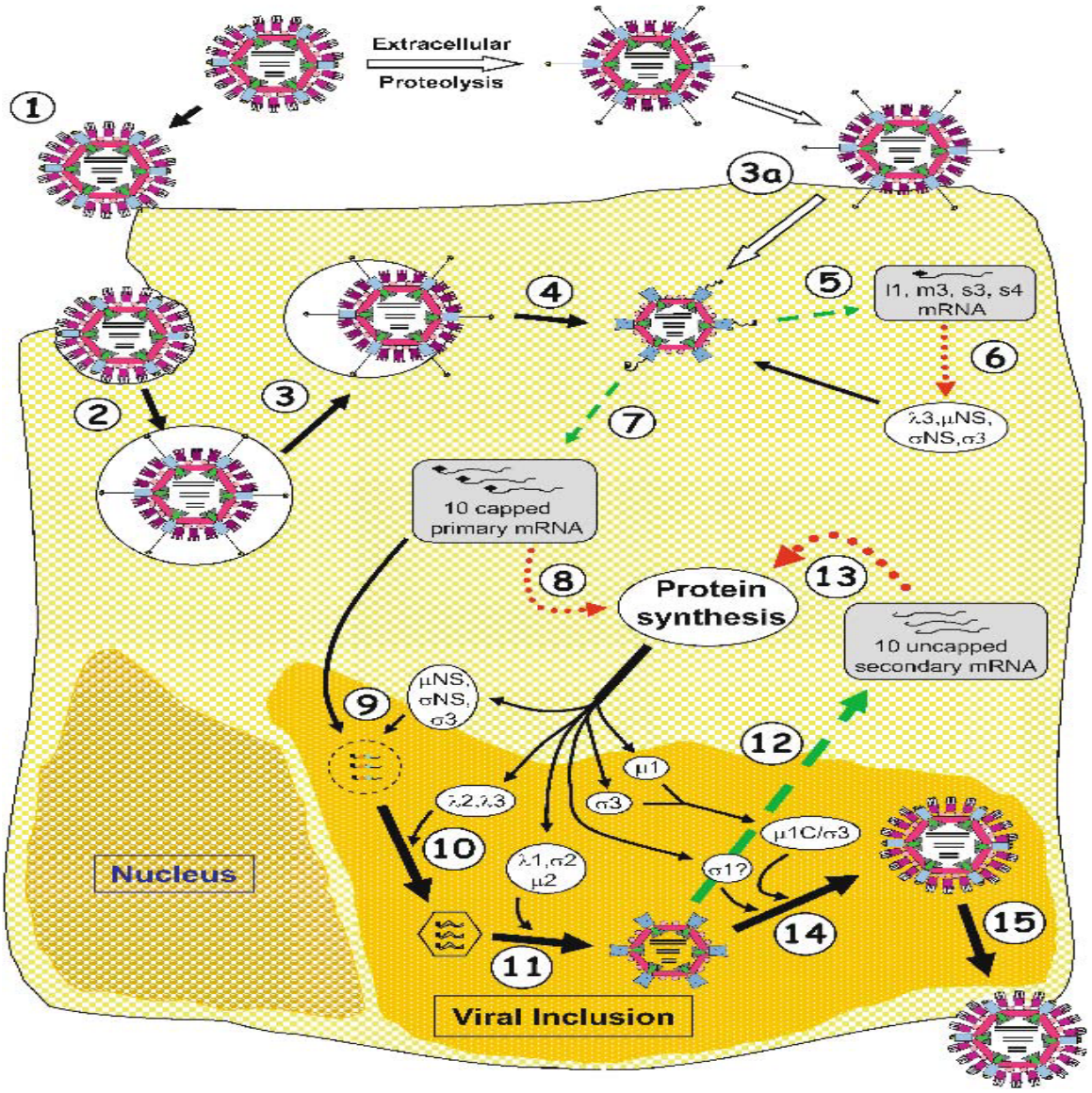


Figure 2. Replication of reovirus. Schematic diagram demonstrating the steps in the replication of reovirus in animal cell.

Source: Reovirus structure and morphogenesis, Coombs, K. M. (2006) with permission.

Once in the cell cytosol, the virus particle undergoes a process of uncoating which is necessary for full activation of the viral transcriptase and leads to the formation of the third form of the reovirus, the core particle. Such process involves removal of the remaining outer capsid proteins and conformational change in λ2 pentamers. The resulting form is the core, the transcription complex that synthesizes mRNA. Genes are transcribed from the minus strand of dsRNA; however, it is thought that only one large (L1), one medium (M3), and two small (S3 and S4) are initially transcribed and translated to proteins λ3, μNS, σNS, and σ3 respectively (Spandidos, Krystal et al. 1976). The products of these initial transcripts stimulate subsequent transcription of the ten segments by the core through unknown mechanism. Translation of these ten genes produces the complete set of viral proteins which then aggregate in the cytoplasm.

The next step comprises the assortment and the generation of dsRNA segments from the ten different mRNA (Morgan and Zweerink 1975). The mechanism of assortment of these different segments into progeny viral particles is yet to be determined. σNS and μNS proteins; however, might be involved in the process (Gillian and Nibert 1998). The nascent core-like particles have the capability to produce mRNA and they further account for the majority of transcription during the replication cycle (Coombs 1996).

The last step in the replication cycle of the reovirus is the assembly of the progeny virion. It involves the incorporation of the ten different segments with the appropriate numbers of each of the eight different viral proteins. Proteins λ1 and σ2 assemble to form the inner capsid shell followed by the association of λ2 proteins. σ1 protein may be incorporated simultaneously due to its structural association with λ2 spikes. The proteins

of the outer shell (μ1 and σ3) bind in form of heterohexamers (Liemann, Chandran et al. 2002) which are accompanied by conformational changes. The double shell is completed when μ1/σ3 heterohexamers associate with the nascent core particles (Chandran, Walker et al. 1999). Several rounds of replication cycle would eventually result in the lysis of cells and release of the progeny virions.

1.4.5 Host range and viral propagation

Reoviruses have one of the widest ranges of susceptible host. It has been demonstrated that reoviruses in addition to humans, infect massive variety of mammalian and avian species. This has been evidenced by either virus isolation or antibody surveillance. Reovirus infection in these animals is manifested mainly by respiratory, enteric and neurological symptoms. Conjunctivitis, rhinitis, and pneumonia in horses, cattle, sheep and pigs comprise the majority of noticed diseases. However, dogs and cats however, may develop any of the three major manifestations. In avian species, the symptoms are mainly enteric.

Owing to its wide host range, reoviruses were found able to grow in different types of cells in vitro. It has been found that L-929 cell (mouse fibroblast) is the appropriate cell line for the propagation of reoviruses for stocks and purification, and for quantitative assays for viral titer. However, reoviruses are also capable of producing cytopathic effects (CPE) in other cell types including HeLa cells (human), human embryonal kidney cells (HEK), and African green monkey kidney cells (CV-1) (Tyler 1999).

1.5 Reovirus Propagation

1.5.1 The demand

The field is now extremely close to a critical tipping point. One randomized, controlled Phase 3 trial is now underway. OncoVEX, an oncolytic herpes virus expressing GM-CSF, is in a Phase 3 clinical trial in patients with advanced metastatic melanoma (comparing intratumoral OncoVEX versus intratumoral GM-CSF protein). JX-594 will also be entering a pivotal Phase 3 trial in 2010. Patients with advanced hepatocellular carcinoma will be randomized to JX-594 or placebo followed by standard sorafenib therapy. Finally, an oncolytic reovirus (Reolysin, Oncolytics Biotech) has received a special protocol assessment by the FDA and is soon expected to enter a Phase 3 trial in patients with head and neck cancer, in combination with chemotherapy. Despite extensive preclinical research programs, the clinical development of OVs has lagged behind, largely due to the fact that large pharmaceutical and venture capital companies have been reluctant to invest in OV programs (Breitbach, Reid et al. 2010).

1.5.2 Challenges

In experimental and clinical trial settings, single administration of reovirus as oncolytic preparation has a dose in the range of 10^7-10^9 PFUs and usually multiple administrations are required to achieve the optimum effect that would result in significant or complete regression of a given tumor (Coffey, Strong et al. 1998; Yang, Senger et al. 2004). The current approach of oncolytic reovirus production is through conventional cell culture. Cell culture maintenance for virus production has direct and indirect costs which would be reflected on the final cost of sales and eventually on the affordability for the

purpose of conducting large-scale experimental and clinical trials. Several studies were performed for the selection of the best yielding type of cell lines.

1.5.3 Literature review

In general, the first step of maximizing virus yield in cell culture systems is to find the most suitable cell line. For example, Barta (Barta, Springer et al. 1984) evaluated two avian and seven mammalian cell lines for the propagation of avian reovirus WVU 2937 and found that chicken embryo liver (CEL) cells formed monolayers quicker, manifested larger plaques, and yielded higher virus titers than chicken embryo kidney (CEK) cells which had been considered the most suitable cell culture for avian reovirus isolation and propagation. They thought that virus-host specificity is not an issue in the suitability for the best yield as VERO cell (mammalian) gave higher titer than CEL (avian). Butler (Butler, Burgener et al. 2000) found that both the specific productivity and the final virus yield of two strains of reovirus (T1L and T3D) were higher in serum-free than in serum-supplemented cultures. Burgener (Burgener, Coombs et al. 2006) concluded that viral productivity was dependent upon the metabolic state of the cells rather than the phase of growth at which the cells were infected. Cultures with lower ATP and total adenylate levels at the time of infection resulted in as much as a 95% reduction in overall viral titer compared to cultures with higher ATP and adenylate. Jung and colleagues (Jung, Behie et al. 2004) determined that the final virus titer was dependent on the input multiplicity of infection (MOI) and the host cell density at the time of infection where a combination of an MOI (multiplicity of infection) of 0.1 and an initial host cell density of $1.0x10^6$ cells/mL in fortified medium resulted in a maximum virus titer, with reovirus titer reaching $4.5x10^9$ pfu/mL. In microcarrier cultures another critical parameter

is likely to be the cell/ bead ratio at infection. Berry (Berry, Barnabe et al. 1999) proposed the feasibility of a culture process for reovirus production based on the infection of Vero cells grown on Cytodex-1 microcarriers to high cell density. They demonstrated that infection at low MOI followed by re-feeding to produce a high cell/bead loading ensures a consistent virus yield per cell and a total viral titer proportional to the maximum cell density in culture.

1.6 The Idea of This Study

1.6.1 Hypothesis

Viruses are obligate intracellular entities in which the cell they infect provides the machinery the viruses need to replicate and produce new progenies. Based on this established concept, it could be hypothesized that the outcome of the virus replication depends on the physiological status of the host cell. The latter is controlled by multiple environmental factors based on the conditions of the culture system. Conceivably, manipulation of these conditions may alter the biological processes and the replication fitness of the host cells and the viruses respectively. Infection of the cells at combination of different culture condition may result in favorable one in term of promotion for higher titer.

1.6.2 Purpose

The objective of the present study was to carefully define the effect of various growth condition parameters on the production of live reovirus, strain T3D in L929 (mouse fibroblast) cell culture and to select the combination that produced the highest virus titer. Six parameters were involved in the study; multiplicity of infection (MOI),

hours post infection (HPI), cell density at infection, cell re-feeding at certain HPI, media pH, and flask size.

2 Materials and Methods

2.1 Materials

2.1.1 Infection

2.1.1.1 Cells

Specifications

L929 (Connective tissue, mouse) clone of strain L was derived in March, 1948 by Earle et al (Sanford, Earle et al. 1948). Strain L was one of the first cell strains to be established in continuous culture, and clone 929 was the first cloned strain developed. The parent L strain was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse. Clone 929 was established (by the capillary technique for single cell isolation) from the 95th subculture generation of the parent strain (ATCC 2010). These cells permit replication of various viruses, such as herpes simplex virus (Garabedian and Scott 1967), sindbis virus (Ito et al., 1989), vaccinia virus (Hruby, Lynn et al. 1980), and reovirus serotype 1, 2, and 3 (Fields, Knipe et al. 2007). It has been the preferred cell type for reovirus because it allows replication to high titer in short time (Jung, Behie et al. 2004).

Nutrition requirements

The base medium for this cell line is Joklik's modified Minimum Essential Medium (MEM) (Eagle 1955; Lockart and Eagle 1959). To make the complete growth medium, it is supplemented with 6% FCS and 2 mM Glutamine (see recipe, Appendix). This medium is formulated for use with a 5% CO_2 in air atmosphere (ATCC 2010). The final ingredients of the media have buffering

components, sodium bicarbonate and Hepes, both keep the media around neutral pH under 5% CO_2 incubation.

In our lab, large volume of double concentration MEM media is prepared by dissolving group stocks; acidic and non-acidic amino acids, vitamins and growth factors; glucose, salts, and Phenol Red (pH indicator) in distilled water. The pH is measured to confirm the intended range of 7.1-7.2 followed by filter sterilization. The ready media is then aliquoted in 1.0 Liter bottles and stored refrigerated at 4°C. Prior to use, the concentrated media is diluted 1:1 with sterilized distilled water and completed with 6% FCS and 2 mM Glutamine.

Maintenance

Cryopreserved stock of L929 cells was initially passaged as described by the manufacturer (ATCC 2010), in which the vial was thawed by gentle agitation in a 37°C water bath then the contents were transferred to a centrifuge tube containing 9.0 ml complete culture medium and spun at approximately 125 g for 5 to7 minutes. The pellet was then resuspended and the contents were added to the culture vessel containing the complete growth medium and incubated at 37°C in 5% CO2 air atmosphere. This passage served as a source for subsequent maintenance (i.e. scale-up) through subculturing in larger monolayer flasks. For attached monolayers, subculturing is performed by rinsing with PBS-EDTA (see recipe, Appendix) followed by trypsinization with trypsin-EDTA (see recipe, Appendix) to detach and resuspend the cells for split into new culture vessels at desired ratio. In our lab (for large-scale maintenance), L929 cells were routinely split daily to 5 x 10^5/mL and maintained in 500 mL spinning flask in a humidified

incubator at 37°C and new flask is prepared every two weeks from monolayer attached in stationary flask. Whenever needed, L929 cells were cryopreserved in complete growth medium described above supplemented with 5% (v/v) dimethyl sulfoxide (DMSO) and stored frozen at -70c.

2.1.1.2 Virus

Stock preparation

Reovirus T3D can be propagated in L929 monolayers or in spinner flasks for large-scale propagation. Purified stocks of reovirus T3D were prepared according to a protocol published by another member in our lab (Berard and Coombs 2009). Briefly, L929 cells were infected at MOI 5 and allowed to incubate at 37°C until 85% cytopathic effect for stationary propagation, or at 33°C for 65 hrs for spinning method. Viruses were harvested by three freeze (-70°C)-thaw cycles for monolayer flasks or by sonication for spinner flasks after concentration by centrifugation. Purification of harvested materials was finally obtained through ultracentrifugation in cesium chloride gradient solution.

Storage

T3D reovirus stocks were kept at 4°C for many months with hardly any loss of infectivity, but the virus looses infectivity at higher temperatures. Large stocks of virus are kept frozen at -70°C in aliquots for years in our lab (Berard and Coombs 2009).

Plates

Infections were performed in 24-well plates (Corning Inc., 3527) in most of the steps except in that of testing the effect of vessel size on the virus titer; different types and sizes of vessels were included in addition to the 24-well plates. These were; 12-well plate (Corning Inc., 3512), 6-well plate (Corning Inc., 3506), 60mm culture dish (Corning Inc., 430166), 100mm culture dish (Corning Inc., 430167), T25 flask (Corning Inc., 430168), T75 flask (Corning Inc., 430720), and T150 flask (Corning Inc., 430823).

2.1.2 Titration

Plate

All plaque assays for determination of virus titer were performed in 6-well cluster plates. 96 well-plates (Corning Inc., 3585) were used for serial dilution of viral harvests.

Virus inoculums

30 µL of harvested material was serially diluted 1:10 up to 10^{-7} in gel saline. From certain dilutions, 100 µL would be added to each well of 6-well titration plate. The inoculums were stored refrigerated at 4°C until the time of use.

Cells

L929 (mouse fibroblast) cell line was used for reovirus titration. These cells were maintained as described earlier (2.1.1.1, pg. 21).

Media-agar overlay

It is a nutritional mixture used to feed cells in the plaque assay. It is composed of media, agar, buffer, and antibiotics. It was prepared by adding equal volumes of melted (62°C) 2% agar and double concentration of commercially formulated (Gibco) 199 completed media (see recipe, Appendix), 0.4 mL of sodium bicarbonate ($NaHCO_3$) per 100 mL of media-agar mixture, and 1 mg/mL of antibacterial (Penicillin-Streptomycin) and 1 µg/mL antifungal (Amphotericin B) (see recipe, Appendix). The mixture was maintained in liquid state by immersion in 62°C water bath and cooled down to around 37°C at the time of use.

Stain-agar overlay

This component was used to facilitate plaques count which appear as clear spots compared to the stained background. It is composed of agar, stain, and phosphate buffer solution (PBS) (see recipe, Appendix). It was prepared by adding 0.8 mL of 2% neutral red stain to each 50 mL of melted (62°C) 2% agar. 2x PBS solution was added in a volume equal to that of the agar. The mixture was maintained in liquid state by immersion in 62°C water bath and cooled down to around 37°C at the time of use.

2.1.3 Cell count and viability

Trypsin

It is a digestive enzyme able to hydrolyze proteins. Trypsin-EDTA was used to detach cell monolayers for count and viability analysis.

Trypan blue dye

It is a vital stain used to selectively color dead tissues or cells blue while live cells or tissues with intact cell membranes are not colored. 0.4% solution (SIGMA T8154) was used at 1:1 ratio with cell suspension for cell viability analysis. This hemocytometer consists of 9 large 1 x 1 mm (1 mm^2) squares. The coverslip is held 0.1 mm off the marked grid by the raised edges of the hemocytometer. This gives each square a defined volume. The total number of cells per ml can be obtained by multiplying the total number of cells in a large square (often the average of the four at corners) by 10^4 (10000).

Hemocytometer

A devise used to count cells in a known surface area. An improved Neubauer hemocytometer (Spencer, 1/10 mm deep, 716828) was used to count L929 cells.

Miscellaneous

15 mL centrifuge tubes (Corning Inc., 430766) were used to spin down the cells using refrigerated centrifuge (Thermo Electron Corp., Ice Central GP8R). Cell count was performed by inverted light microscope (Nikon, Eclipse TE2000-S), and digital camera (Canon, Power Shot A700) was used to capture images of microscopic fields when indicated.

2.1.4 Manipulation and measurement of media pH

Acid and alkali

Hydrogen chloride (HCl) and sodium hydroxide (NaOH) were used to respectively lower or raise the pH of the completed MEM media to certain values.

PH meter

An electronic instrument used to measure the pH (acidity or alkalinity) of a liquid. It typically consists of a special measuring probe (a glass electrode) connected to an electronic meter that measures and displays the pH reading. (Fisher Scientific, Accumet AB15) was used to monitor the achievement of certain pH values by addition of HCl or NaOH. Alternatively, pH strips (EMD Chemicals, ColorpHast, 9583) were used to measure pH inside culture wells.

Filters

150 mL bottle top 0.22 µm filter (Corning Inc., 430624) was used to sterilize the media aliquots after pH adjustment.

Magnetic stirrer

A laboratory device that employs a rotating magnetic field to cause a stir bar immersed in a liquid to spin very quickly, thus stirring it. (Corning Inc., PC-351) was used to continuously mix the HCl or NaOH added to the media.

2.2 Procedures

2.2.1 Growth curve

First, a growth curve was setup to determine the optimal time (hour post infection; HPI) and virus-cell ratio (multiplicity of infection; MOI) for maximal plaque forming unit (PFU) yield. The infections were conducted at six MOI points 0.01, 0.1, 0.25, 0.5, 1.5, and 5.0 and harvested at seven HPI intervals 0, 1, 12, 24, 48, 72, and 96. The infections were carried out in subconfluent (~ 95%) cell density at the time of infection according to the established protocol in our lab and the HPIs counted after virus adsorption to cells as described in the infection section below. Seven 24-well plates

representing the seven HPIs were seeded overnight with L929 cells at 2.5 x 10^5 per well in 0.5 mL suspension from the spinning flask and incubated at 37°C in 5% CO_2 with one extra well per plate for cell count. Prior to cell count and infection, wells were assessed for confluency and checked for contamination. The extra well in each plate was processed for cell count according to the protocol described later (2.2.9, pg.34). The average of the seven wells was determined, and the amount of plaque forming units (PFUs) for each MOI value was extrapolated accordingly. Infections were performed and then stopped at the assigned HPIs for harvest as described later in the infection and harvest protocols respectively. The harvested materials were then assayed for reovirus T3D titers by plaque assay as outlined in the titration protocol later. The titers were plotted against the HPI intervals for each MOI.

2.2.2 Cell density

The second parameter included in this study was the effect of cell density (% confluent) at the time of infection on the virus yield. This parameter would test the hypothesis that cellular growth kinetic as one of the indicators of the physiologic status of the host cell. For this purpose, infections were aimed to be conducted at different stages of cell growth. Therefore, 24-well plates were seeded with L929 cells for several density levels, 50, 75, 95 (default), 105, 125, 150, and 200% at the time of infection. Infections were performed with best combination of MOI and HPI that gave the highest titer in the first step (the growth curve). Prior to infection, the cell monolayers were assessed microscopically for the assigned densities and cell counts were determined for correlation. Cell counts, reovirus titers (PFU/mL), and reovirus yields (PFU/cell) were plotted against the assigned densities.

2.2.3 Media volume

The third step in this work was to investigate the effect of overlay medium in terms of the volume to surface area (or cell number) ratio and the re-feeding (medium replenishment). This parameter was included upon the assumption that cell physiology might be influenced by the volume of media in certain pattern, taking into consideration the nutritional abundance and replenishment, dilution of solutes, and the distance between the monolayer and the surface of overlaying media (where the gas exchange occurs) as potential contributors. Three volumes of 0.25, 0.5, and 1.0 mL, corresponding to the ratios 0.125, 0.25, and 0.5 mL per 1 cm^2 surface area (~ 2.5 x 10^5 of cells) or 1.3, 2.6, and 5.2 mm depth of cell culture in 24-well plate, were used to overlay the infection wells after virus adsorption. One set had the initial overlay remained throughout the incubation period while the other set had approximately two thirds of the initial overlay replaced by new medium. The MOI, HPI, and cell density of infection used were the conditions that gave the highest titers in the previous steps.

2.2.4 Flask size

The fourth factor studied was the size of infection vessel. The idea was to run the infection in different vessel sizes to examine how the reovirus titers might be affected and to consequently explore the responsible causes. The thought implied in considering this parameter is that different sizes and types of reaction vessels have variable spacial dimensions which may result in different air atmospheres and evaporation rates. Eight different sizes and types were used, 24-well (2 cm^2), 12-well (4 cm^2), and 6-well (9.5 cm^2) plates; T25 (25 cm^2), T75 (75 cm^2), and T150 (150 cm^2) flaks; 60mm (21cm^2) and 100mm (56cm^2) Petri dishes. The amount of cells seeded was scaled to the ratio of 1.25 x

10^5 cells per cm^2 surface area in 0.25 mL media suspension. The volumes of virus inoculums (at the specified MOI) and media overlays were also scaled to the appropriate ratios (**Table 1**).

2.2.5 Media pH

Last factor included in this study was the pH of the media. As established, mammalian cells have narrow range of tolerable (and even narrower for optimal) pH in the growth environment. However, it is not known whether the buffering ingredients in the media could – upon exposure to 5% CO_2 air atmosphere – adjust for the optimal pH or not and how such condition might impact the cell survival and reovirus titer. Therefore, infections were overlaid with media of different pH values. The initial plan was to start with range of 6.0 to 8.5 with half point intervals in addition to the default in our lab (7.2-7.5). This range was subjected to expansion to reach determining cut-off limits in both (acidic and basic) sides based on the results from the initial range. Adjustment of normal media to different pH values was performed as described below. Similarly, the infection specifications were fixed to the best of the preceding parameters. Plaque assay was performed for reovirus titer half way through and at the end of the incubation period. Cell count and viability analysis were done to assess the effect of the pH on cell growth and viability.

2.2.6 Reovirus T3D infection of L929 cells

L929 cells were seeded overnight in completed MEM media for the required density. Reovirus T3D inocula were prepared by dilution of the virus stock in gel saline so that the PFU/cell corresponds to each MOI is maintained in a volume of 50 µL per well of 24-well plate size (~2.0 cm^2 surface area). The inoculum volume was scaled –

Size, type, and surface area (cm^2) of Infection Vessel	Amount of Plated Cells (cells/vessel)	Volume of overlay Media (mL)	Volume of virus inoculum (mL)
24-well (1.9)	2.5×10^5	0.5	0.05
12-well (4.0)	5.0×10^5	1	0.1
6-well (9.5)	1.2×10^6	2.4	0.24
60 mm-Petri (21.0)	2.6×10^6	5.2	0.52
100 mm-Petri (56.0)	7.0×10^6	14	1.4
25 T-Flask (25.0)	3.12×10^6	6.24	0.624
75 T-Flask (75.0)	9.37×10^6	18.74	1.874
150 T-Flask (150.0)	1.87×10^7	37.4	3.74

Table 1: Scaled specifications of reovirus T3D infection of L929 cells in different vessels. Amount of cells plated and volumes of overlay media and virus inoculm were scaled to the ratio of the surface area to that of 24-well (the one used in all of the other parameters included in the work). Before infection, monolayers in different vessels were assessed microscopically for comparable sub-confluent density (within 5% range of variation).

proportionally to the surface area – for other sizes. All infections were conducted in 24-well plate except when different sizes were tested. The seeding medium was removed and cell monolayer was infected by the inoculum. The viruses were allowed to adsorb to the cells for a minimum of one hour at room temperature before they were overlaid with completed MEM media supplemented with Amphotericin B and Penicillin-Streptomycin in a volume of 0.5 mL per well of 24-well plate size. Similarly, the overlay volume was scaled – proportionally to the surface area – for other sizes. The infection vessels were incubated at 37°C in 5% CO2 for the required HPI time.

2.2.7 Reovirus harvest

This procedure refers to the release of reovirus particles from L929 cells in the culture vessel by mechanical destruction of the cells through repeated cycles of freeze and thaw. The vessel was tightly sealed by tape, placed in deep freezer (-70°C) for couple of hours, and removed for thaw at room temperature. The cycle was repeated three times and the resulted lysate was stored refrigerated at 4°C until the time of titration.

2.2.8 Titration

Reovirus T3D titer in this study was determined by the conventional plaque assay in L929 cells. Harvested samples were 1:10 serially diluted in gel saline and 100 uL from 10^{-3}-10^{-7} dilutions were inoculated into L929 cell monolayers in duplicate in 6-well plates. After adsorption for one hour with intermittent rocking every 15 minutes, wells were covered with 3 mL of media agar overlay and allowed to solidify for 15 minutes at room temperature. The plates were incubated at 37°C and 5% CO_2 for 3 days before another 2 mL of media agar overlay was added to the wells. After 2 additional days of incubation, the wells were stained with 2 mL of neutral red-agar overlay and remained

incubated. The plaques were counted with the aid of bright lamp within 18-20 hours after the staining. Dilutions with 20-100 plaques were used to calculate the virus titer by multiplying the number of plaques by the positive reciprocal of the dilution. The reovirus titers were represented as PFU/mL by multiplying by 10 to correct for the $1/10^{th}$ volume used for inoculation. Alternatively, the reovirus yields per cell (PFU/cell) were extrapolated by dividing the titer over the cell count (cell/mL) at the time of infection.

2.2.9 Cell count and viability

First, overlay media was removed and the L929 monolayer was then washed once with PBS-EDTA. An appropriate amount of Trypsin-EDTA was added to the cell monolayer and allowed to react for around 5-10 minutes at 37°C. As the monolayer started to detach, completed MEM was added to inhibit the action of the trypsin and resuspend the detached cells. For cell count, the hemocytometer was loaded with the cell suspension and the average cell count of the four corner (large, 1 mm^2) squares of the grid was determined. The total number of cells per ml was then determined by multiplying the average number of cells by 10^4 (10000). For cell viability, the cell suspension was spun for five minutes at 1000g. The cell pellet was resuspended in PBS and a mixture of the cell suspension and Trypan blue dye 0.4 % solution at 1:1 ratio was prepared. The mixture was gently mixed and allowed to stand for 3-5 minutes before it was loaded into the hemocytometer for count. Cells at the four corner squares were counted either as live if they were grayish (dye exclusion) or dead if they were bluish (dye inclusion). The viability percent was calculated by dividing the live cells over the total (live and dead) cells.

2.2.10 Manipulation and measurement of media pH

Normal MEM media was aliquoted into appropriate size of bottles. The electrode of the pH meter was placed inside the bottle containing the media and either 1M Hydrochloric acid (HCL) or Sodium Hydroxide (NaOH) was slowly dropped into the solution in amount not exceeded 4% of the total volume of the media aliquot until the required pH value was reached as reflected in the electronic reader of the pH meter. The adjusted aliquots were then filtered by 0.22 um filter and stored refrigerated for no longer than 2 weeks for pH stability reason. Due to small volume, measurement of media pH in the 24-well plate was performed with pH paper (which has a scale of 0.5 intervals) according to the product instruction.

3 Results

3.1 Growth Curve

I initially examined progeny production over a period of four days after sub-confluent cells were infected at increasing virus to cell ratios (MOIs of 0.01, 0.1, 0.25, 0.5, 1.5, and 5 PFU/cell). Initial titers (≤ 12 HPI) correlated with starting inoculated titer. Titers increased by at least two $\log_{10}$ by 24 HPI. Subsequent increases in titer were dependent upon input MOI. From 12 to 24 HPI, the titer of the input MOI 0.1 increased by 2.7 $\log_{10}$ compared to 2.1 $\log_{10}$ of MOI 5.0. Additionally, subsequent titer increases for lower MOIs were always higher than that of higher ones as demonstrated in the graph by steeper trends (**Figure 3**). The plotted titers represent the average of three repeats; however, error bars are not shown due to extensive confusing overlaps. They could be found in the comprehensive table (**Table 2**). The highest average titer was obtained at 96 HPI at MOI of 0.1, 2.83×10^9 ($\pm$ 6.43×10^8) PFU/mL. Thus this MOI was chosen for evaluation of other parameters in the next steps.

3.2 Cell Density

Different cell densities were next examined. For this component of the study, we plated different numbers of cells. Thus, a value of "150%" indicates that approximately 50% more cells were added to wells compared to the number pre-determined to produce a 95-100% confluent monolayer. And, a "200%" monolayer would not necessarily contain twice as many cells as a "100%" monolayer, because cells would have stopped growing through contact inhibition (**Figure 4**), but the cells would be expected to have stopped replicating sooner that "150%" cells, which would have stopped replicating

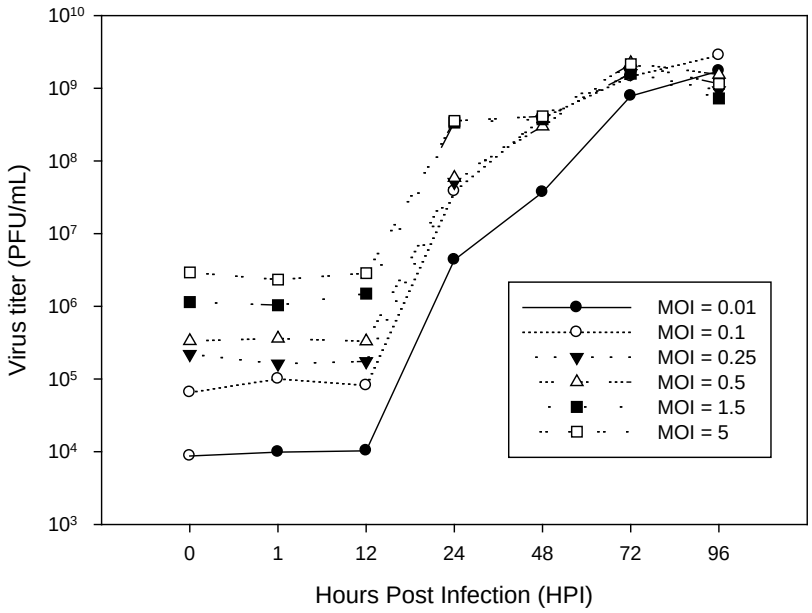


Figure 3. Reovirus T3D growth curve in L929 cells. Sub-confluent (95%) monolayers in 24-well plates infected at 6 MOIs and harvested by 3 freeze-thaw cycles at 7 HPIs. The harvested materials were assayed for total PFU by conventional plaque assay in L929 cells. The titers represent the arithmetic mean of three repeats.

Multiplicity of Infection (MOI)		Hours Post Infection (HPI)						
		0	1	12	24	48	72	96
0.01	pfu/ml	$8.73x10^3$	$9.90x10^3$	$1.03x10^4$	$4.35x10^6$	$3.71x10^7$	$7.82x10^8$	$1.72x10^9$
	SD	$1.33x10^3$	$1.44x10^3$	$1.44x10^3$	$3.06x10^6$	$2.40x10^7$	$6.25x10^8$	$8.69x10^8$
0.1	pfu/ml	$6.52x10^4$	$1.00x10^5$	$8.11x10^4$	$3.81x10^7$	$3.90x10^8$	$1.45x10^9$	$2.83x10^9$
	SD	$3.05x10^4$	$5.63x10^4$	$5.63x10^4$	$2.95x10^7$	$3.13x10^8$	$1.09x10^9$	$6.43x10^8$
0.25	pfu/ml	$2.19x10^5$	$1.62x10^5$	$1.75x10^5$	$5.09x10^7$	$3.72x10^8$	$1.60x10^9$	$9.27x10^8$
	SD	$1.11x10^5$	$1.01x10^5$	$1.01x10^5$	$3.42x10^7$	$2.09x10^8$	$1.35x10^9$	$1.73x10^8$
0.5	pfu/ml	$3.32x10^5$	$3.59x10^5$	$3.29x10^5$	$5.83x10^7$	$2.98x10^8$	$2.22x10^9$	$1.53x10^9$
	SD	$1.88x10^5$	$1.94x10^5$	$1.94x10^5$	$3.84x10^7$	$1.16x10^8$	$2.42x10^9$	$1.19x10^9$
1.5	pfu/ml	$1.14x10^6$	$1.03x10^6$	$1.49x10^6$	$3.36x10^8$	$3.73x10^8$	$1.58x10^9$	$7.22x10^8$
	SD	$8.25x10^5$	$7.67x10^5$	$6.77x10^5$	$2.48x10^8$	$1.20x10^8$	$1.52x10^9$	$2.37x10^8$
5	pfu/ml	$2.91x10^6$	$2.32x10^6$	$2.85x10^6$	3.5410^8	$4.10x10^8$	$2.13x10^9$	$1.14x10^9$
	SD	$1.16x10^6$	$1.13x10^6$	$1.13x10^6$	$2.47x10^8$	$1.43x10^8$	$1.21x10^9$	$8.06x10^8$

Table 2: Reovirus T3D titer in L929 cells. Sub-confluent (95%) monolayers in 24-well plates infected at 6 MOIs and harvested by 3 freeze-thaw cycles at 7 HPIs. The titers represent the arithmetic mean of three repeats.

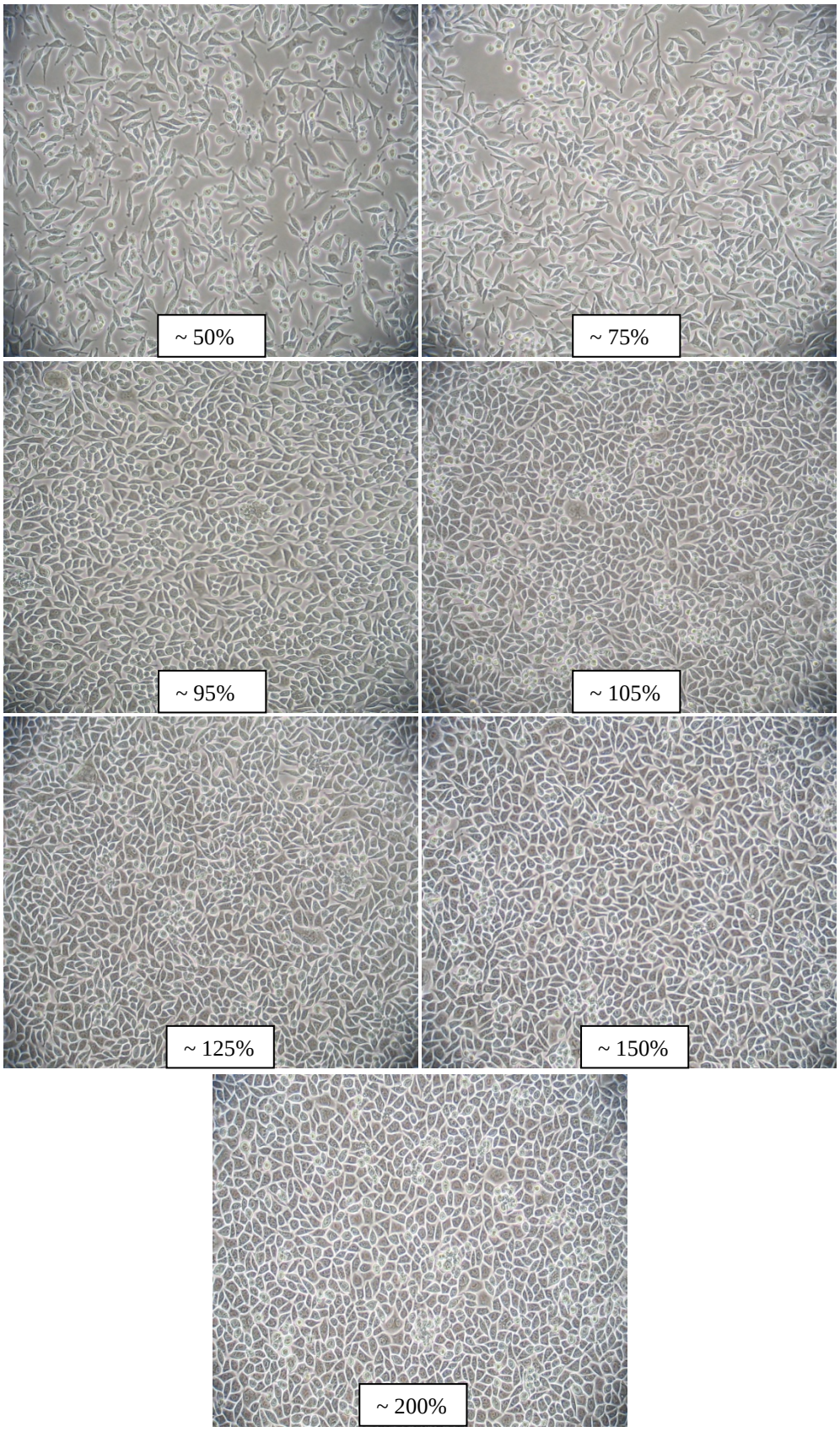

~ 50%
~ 75%
~ 95%
~ 105%
~ 125%
~ 150%
~ 200%

Figure 4. Density of L929 cells at the time of infection. L929 cells were seeded overnight in 24-well plate at various concentrations of cells in fixed volume of media. The amount of cells plated for each of the aimed density was proportional to that of 95%, standard infection density for propagation of reovirus in L929 cells.

sooner that "100%" cells. Prior to infection, the intended densities were checked through microscopic assessment and cell count (**Figure 5**a). Average virus titers after cells were infected at different cell densities are shown (**Figure 5**b). Similar to the first step, the difference in absolute amount of virus produced is minimal and within 1 $\log_{10}$. The highest titer obtained was from cells at 95% confluent at infection, although higher PFU/cell values were attained when cells were infected at lower confluency. Consequently, subsequent parameters were conducted with cell density of 95% confluency (**Figure 5**c).

3.3 Media Volume and Re-feed

It might be expected that media replacement would replenish nutritional elements and hence enhance virus production. However, to my knowledge, this has not been formally tested. Under our scheme, T3D replicated to approximately 1 x 10^9 PFU/mL when media were not replenished and irrespective of the overlay volume. However, the titer was 2-4 folds higher and was highest (~ 4x10^9 PFU/mL) when 24-well monolayers were covered with 0.25-0.5 mL (corresponds to 1.3-2.6 mm) overlay and two-third of the initial overlay was replaced with fresh media (**Figure 6**).

3.4 Flask Size

Having established that maximal reovirus T3D production resulted from infecting 95% confluent cells at an MOI of 0.1 PFU/cell, overlaying cells in 24-well with 2.6 mm of overlay media, and replenishing media at 48 HPI, I next examined the effects of vessel size. Media volume was scaled such that all cells were covered with 0.125 mm of media per cm^2 surface area. Interestingly, there was a size-dependent pattern, such that virus

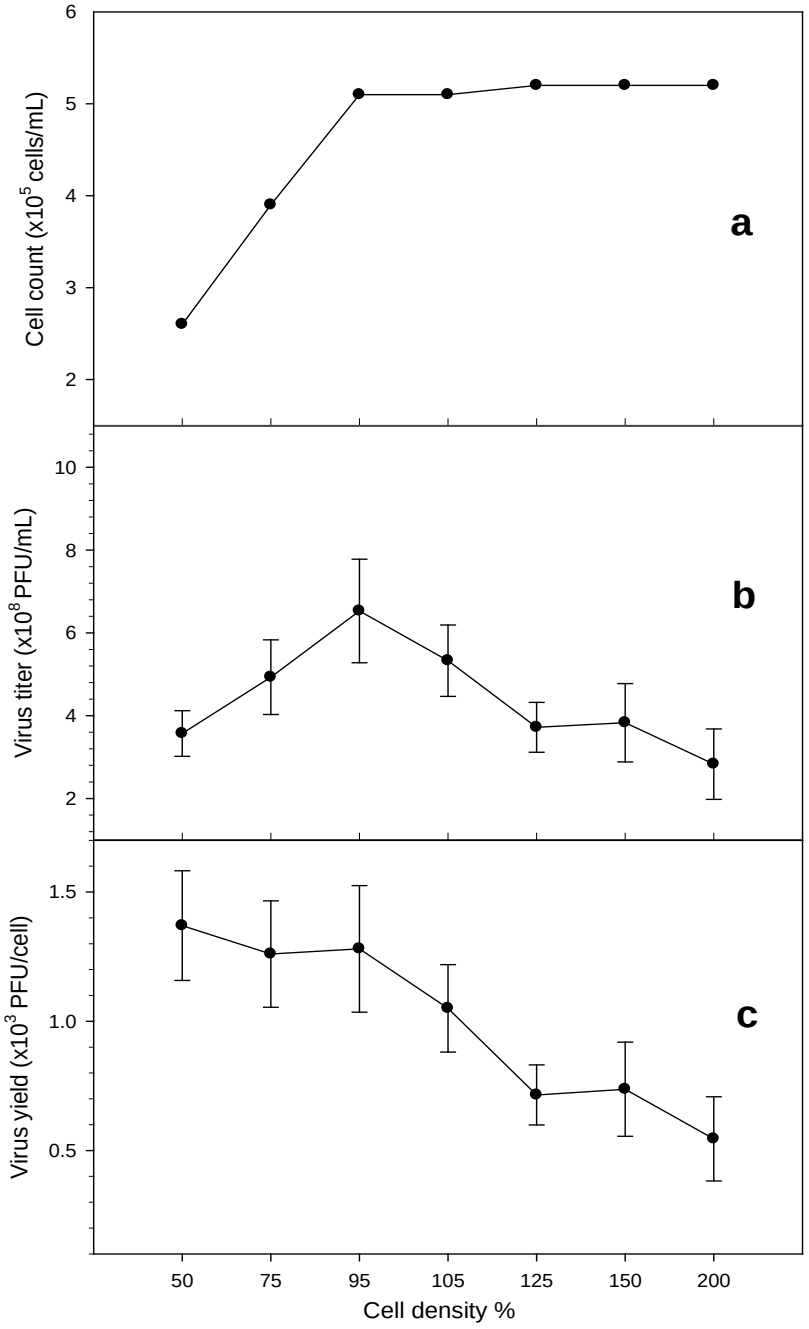


Figure 5. Cell counts, virus titers, and virus yields at 7 cell density percentages. L929 cells seeded overnight in 24-well plates for the assigned densities. a) Cell counts at the time of infection. b) Reovirus T3D titers at 96 HPI at MOI of 0.1. c) Reovirus T3D yields per cell.

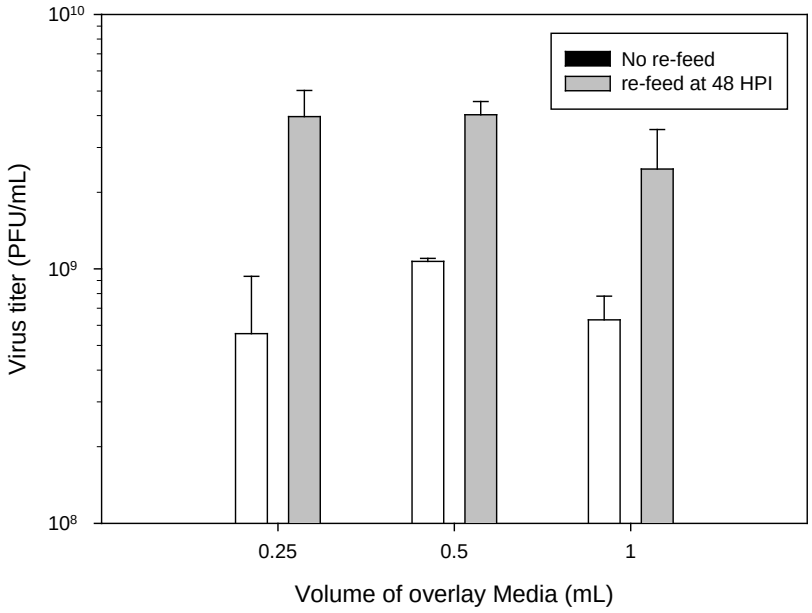


Figure 6. The effect of overlay media volume and re-feeding on reovirus titer in L929 cells. Sub-confluent (95%) monolayers in 24-well plates were infected with reovirus T3D at MOI of 0.1 and overlaid with different volumes of media. The media overlay was either kept throughout the incubation (no re-feed) or 2/3 was replaced with fresh media (re-feed) at 48 HPI.

titers decreased as the flask size increased (**Figure 7**). Thus, titers obtained from ~ 2.0 cm^2 monolayers in each 24-well were approximately 2-fold higher than titers obtained from 150 cm^2 monolayers in T150 flask. However, when these titers were corrected for the lost media due to evaporation, the range (difference between highest and lowest values) narrowed by 65 % from 3.2 x 10^9 to 1.13 x 10^9 (from 1.8 to 0.6 fold difference).

3.5 pH of the Media

As a final component of this study, I examined the effects when media at different pH values was added at the beginning of infection. Interestingly, I found that reovirus production from L929 cells tolerated a broad range of pH, with ~10^9 PFU/mL produced at values of starting pH between 6.0 and 11. However, this range was equivalent to 7.0 and 8.5 respectively due to the action of the buffering system upon exposure to the CO_2 in the incubator (**Figure 8**a). Hence, the latter (7.0-8.5) was considered the actual maximal productive range because virus production was reduced approximately 100 fold under more acidic starting conditions (4.5, 5.0, and 5.5 which were buffered to pH 6.5) and was undetectable at more basic starting conditions (12.0 and 13.0 which were buffered to pH 9.0) (**Figure 8**b). Cell count and viability were consistent with the virus titers with some exceptions (**Figure 8**c).

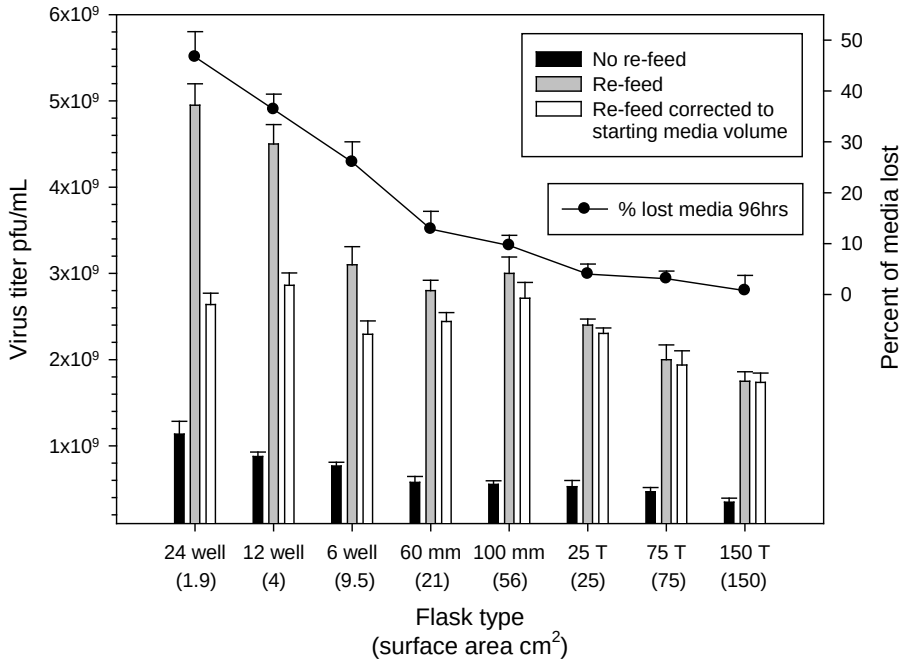


Figure 7. Reovirus T3D titers and percents of media loss at different flask sizes. Sub-confluent L929 cells were infected with reovirus T3D at MOI of 0.1 and harvested at 96 HPI for titrations. Media volumes were measured at 0 and 96 HPIs, the percent of lost media is plotted (scatter line). Titers of the re-feed set were corrected for the lost media (white bars).

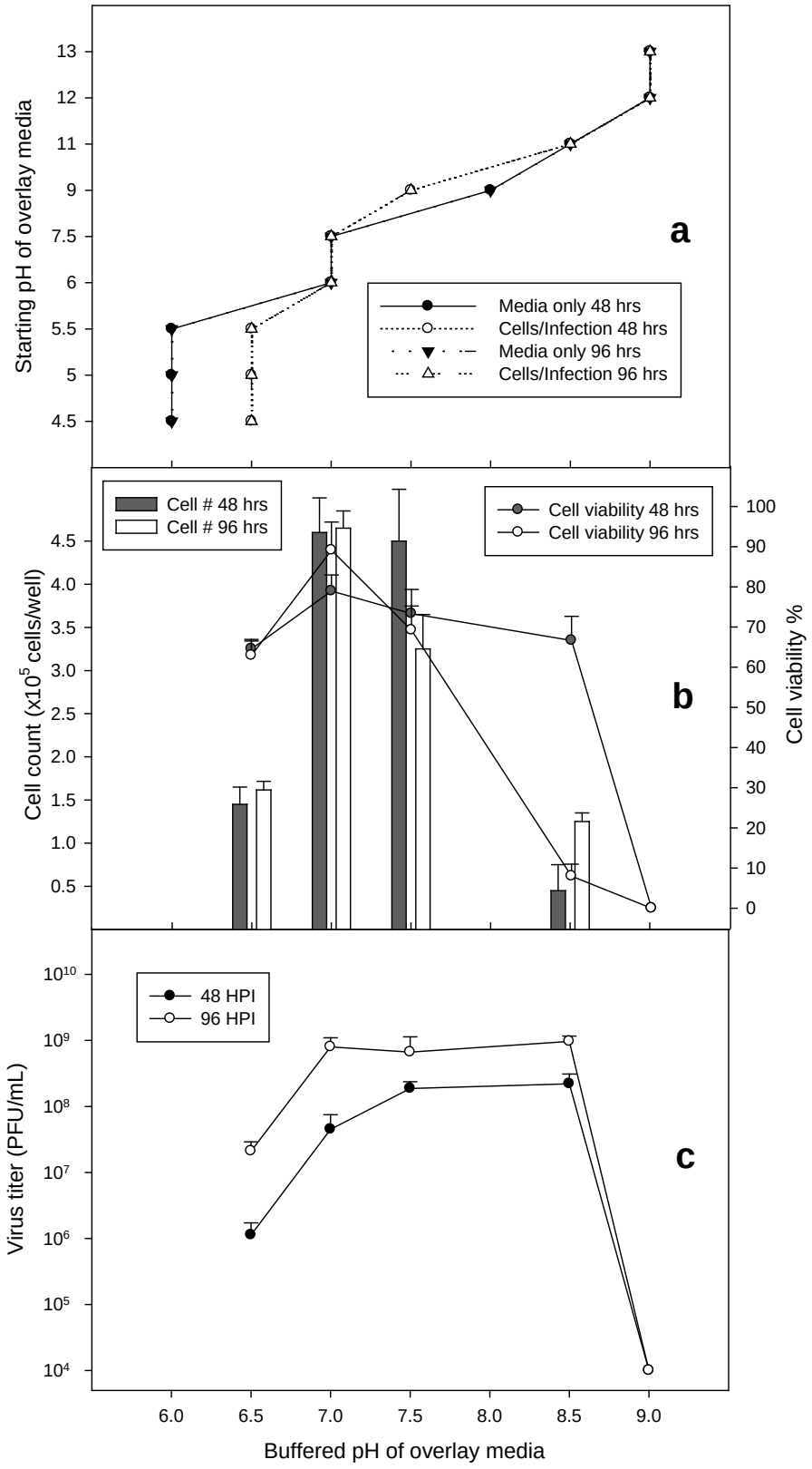

Starting pH of overlay media
13
12
11
9
7.5
6
5.5
5
4.5
a
Media only 48 hrs
Cells/Infection 48 hrs
Media only 96 hrs
Cells/Infection 96 hrs
Cell # 48 hrs
Cell # 96 hrs
Cell viability 48 hrs
Cell viability 96 hrs
Cell count (x10^5 cells/well)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
b
100
90
80
70
60
50
40
30
20
10
0
Cell viability %
48 HPI
96 HPI
Virus titer (PFU/mL)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
c
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Buffered pH of overlay media

Figure 8. Effect of overlay media pH on the cells and virus titer. a) pH drift in 5% CO2 incubation. 0.5 mL per 24-well of Media with assigned starting pH were incubated either alone or overlaying L929 cells or reovirus T3D infected L929 cells. pH was measured at 48 hrs and 96 hrs incubation. b) Effect of overlay media pH on cell growth and viability. L929 cells were seeded overnight in standard pH (7.2) in 24-well plates. Sub-confluent monolayers were then overlaid with media of starting pH range of 4.5-13. pH was measured and wells were trypsinized for cell count and viability at 48 hrs and 96 hrs. c) Sub-confluent monolayers were infected with reovirus T3D at 0.1 MOI and overlaid with media of different starting pH values. At 48 and 96 HPI the pH was measured and the sets were harvested for virus titration by plaque assay in L929 cells.

4 Discussion

In this study, I tried to enhance the production of mammalian reovirus (T3D) in cell culture system through manipulation of certain growth condition parameters that are thought to impact cell physiology and virus replication fitness and consequently the infection progress and virus yield. The study was performed as planned in which the best point in the range of each parameter was selected in testing the subsequent parameters. The difference between the lowest and highest point was as low as 0.41 $\log_{10}$ in the cell density parameter and as high as 2.1 $\log_{10}$ in the pH one.

4.1 Growth Curve

The growth curve of mammalian reovirus (T3D) in L929 cells revealed that lower MOIs might be preferred for high virus production than higher MOIs. This finding suggests that an optimum rather than concentration dependent virus-cell ratio is required for the highest possible virus progeny production. In average as well as in all replicates, the final titers were comparable; however, the MOI of 0.1 showed the most sustained steep in the growth curve graph as the incubation extended to 96 HPI. At 48 HPI, the titer of 0.1 MOI was comparable to all of the higher MOIs, in which the highest MOIs 1.5 and 5.0 seemed to be at transient plateau. After 72 HPI, the titers of high MOIs (0.25, 0.5, 1.5 and 5.0) started to decline, while those of low MOIs (0.01 and 0.1) were increasing. Similar finding was obtained by Grande (Grande and Benavente 2000) with avian reovirus S1133 in chicken embryo fibroblast (CEF) cells. From these results, it could be deduced that inoculating one PFU in cell culture would have the same or higher titer as five hundreds PFUs. Such finding might be significant in the pharmaceutical-scale manufacturing of the reovirus T3D.

The biological threshold of the cells and viruses competition for replication machineries might possibly explain such observation. However, similar finding might not be reproducible with either different cell line and/or virus strain as Berry (Berry, Barnabé et al. 1999) obtained higher titer at MOI of 5.0 than at MOI of 0.5 with reovirus T1L in Vero cells but demonstrated comparable observation to ours with similar strain (T3D).

None of the previous studies have demonstrated any considerable increase in the titer after 96 hrs of incubation, this holds true for reovirus T3D in stationary cell culture of L929 cells in particular as the same strain required over 150 hours incubation to reach the maximum titer in Vero cells in a study by Berry (Berry, Barnabé et al. 1999). Such findings come from the established fact that each virus (species/strain) / cell line has peculiar interactions during the infection cycle which reflect the outcomes, including the virus titer. Perceivably, growth curve could provide useful information on the pattern of virus replication over time and its relationship with the virus-cell ratio. Prior knowledge of such information is required in many settings of virus-host related studies.

In industrial manufacturing of therapeutical reovirus, both the amount of input virus inoculum and the time of the production cycle constitute important economical concerns. It would be the target of the manufacturer to produce the maximum output with the minimal possible input; profit paradigm but also assurance of meeting the demand. Similarly, the time of production cycle would be aimed at the shortest possible for maximum efficiency; not only to maximize the produced units per operation time, but also to minimize the contamination rate (longer incubation may result in higher contamination rate).

4.2 Cell Density

In most of the studies that investigated the production of reovirus in cell cultures, the cell density at the time of virus infection was fixed at subconfluent (~95%) in stationary systems and its equivalent number of cells in suspension system against the variable parameters tested (Barta, Springer et al. 1984; Jung, Behie et al. 2004; Burgener, Coombs et al. 2006). The feasibility of obtaining certain density (within narrow and consistent range) varies among cell types, based in part to the duplication rate and the contact inhibition characteristic. As shown in the cell count plot (Figure 4a), predictive cell density and count correlation were relatively easy to obtain with L929 cells through reciprocal scaling (with minor adjustment) of amount of cells seeded.

Though at lower densities the cells are assuming a high growth rate and at higher densities have more cells (hence, redundant machinery resources), neither of these was advantageous to the total virus titer compared to the subconfluent (~95%) within one $\log_{10}$ range. Similar findings were reported by Jung (Jung, Behie et al. 2004) (though in spinner flask protocol) in which higher titer obtained in amount of cells corresponds to ~95% than either in half or double the amount of cells correspond to lower and higher densities.

The accelerated growth rate of cells at lower than subconfluent densities was associated with higher virus yields (PFU/cell) compared to lower virus yields in decelerated growth rate of cells at higher than confluent densities. It could be suggested that cells at enhanced growth state – which yielded more viruses – would reach their maximum virus production and experience lysis (an established mechanism of reovirus

killing of transformed cells) prematurely as evidenced by microscopic examination, and therefore fewer productive cells remain until the end of the incubation.

The higher the amount of cells plated (as those aimed for >95% in our study), the earlier they become confluent and presumably, the longer they were present at minimum physiological activities related to cell proliferation. Therefore, cells at slowed growth state – which yielded fewer viruses – were able to produce total titer close to that of subconfluent (~95%) if allowed for longer incubation. Other explanation however, states that when cells plated at high density the nutrient elements would be consumed at faster rate and such would destine the cells to stationary phase earlier compared to low density (Butler 2004).

It is however not known if there is a virus species/strain / cell line peculiarity as with the growth curve, in which the maximum titer for each virus species/strain / cell line combination might be obtained in different MOI / HPI values as observed in different studies. However, it could be claimed (at least based on the available studies that showed similar relationship) that cell density might have relatively similar effect on any virus / cell culture system that is conducted in using the same methodology. Taking into account the minimal difference in the total titers, it might be stated then that comparable maximum titer could be obtained with half the amount of cells and virus inoculums, another advantage in pharmaceutical-scale manufacturing. However, in these settings, the quality of the produced viruses has to be critically assessed to ensure their efficacy.

4.3 Media Volume

The availability of essential elements contained in the growth medium in measured amount and scaled ratio is a mainstay for the maintenance of the biological

processes of cells in a culture which in turn may affect the progression of viral infection (Gstraunthaler, Seppi et al. 1999; Jung, Behie et al. 2004). In examining the effect of the ratio of overlay media to the well size (or amount of cells) on the production of reovirus T3D in L929 cells, I found that the maximum titer was obtainable using half (0.25 mL/24-well) the volume of the overlay media commonly used (0.5 mL/24-well). Similarly, overlaying with twice the established optimum volume resulted in equivalent titer. Considering the dilution factor, such comparable titers may indicate different production capacity among the three ratios used. Cells overlaid with half or twice the optimum volume might have produced fewer (due to nutrition depletion) or more (due to nutrition opulence) viruses respectively, yet the dilution factor rendered them falsely similar.

Alternatively, it could be suggested that the peak titer obtained after time intervals of steady increment is influenced in part by the amount of progeny viruses inside the cells and maybe to a lesser extent of that released into the media. Such, may explain the dilution dependent titers of the supernatant at 48 HPI (**Figure 9**) (in which the cells were seemingly at similar log phase) and relatively similar total titers at the peak (96 HPI) which might be attributed to fixed threshold of the cells. However, this interpretation does not explain the 2 to 4 fold increase in the titer after most of the media was replaced with fresh one amid incubation time. It might be possible to suggest that the amount of released viruses might also be involved in the achievement of the peak titer. Dilution of either the suspended viruses (although the removed portions at 48 HPI had approximately only 0.1 % of the final titer at 96 HPI) or the metabolic byproduct may have promoted higher titer over the last 48 hrs. Regardless of the underlying mechanisms, these results

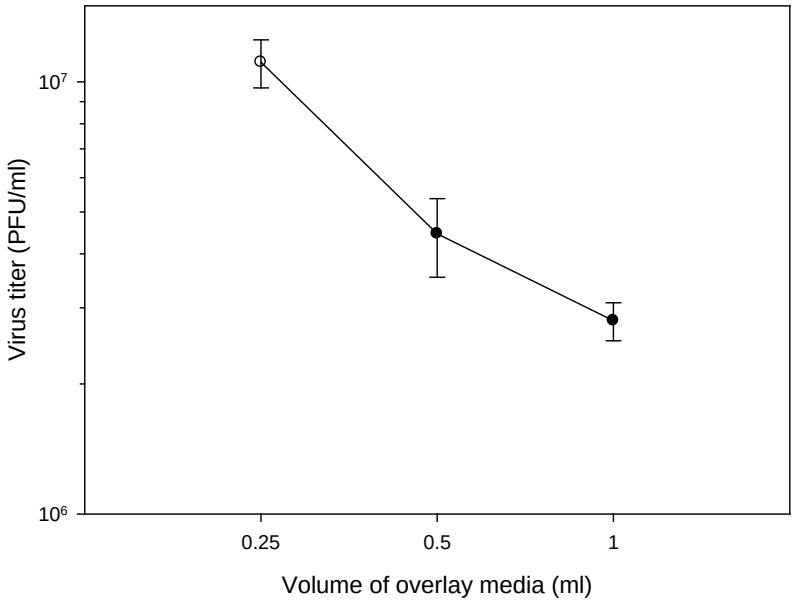


Figure 9: Reovirus titer at 48 HPI in wells overlaid with different volumes of media. Sub-confluent (95%) monolayers in 24-well plates were infected with reovirus T3D at MOI of 0.1 and overlaid with 0.25, 0.5, and 1.0 ml of media. At 48 HPI, 2/3 of the media were removed and assayed for reovirus titer.

further corroborate the effect of the nutritional abundance on the production capacity of reovirus T3D from L929 cells.

Alternatively, the depth of media; the distance between the cells and the media surface where the gas exchange occurs, might constitute a factor, yet it was not assessed in this step as the eventual afflicted parameter is the pH. The media used (pH of 7.2) is strongly buffered around neutral pH in 5% CO_2 and 37°C incubation condition and therefore the extent of drift in the media pH among the different depths would expected to be minor.

From manufacturing point of view, it would also be profitable to ensure the usage of lowest possible volume of media for maximum production.

4.4 Flask Size

Even at the right amount and optimum ratio of the parameters discussed so far, there was a noticeable variation in the titers of infection conducted in different sizes and types of reaction vessel though a scaled ratio was maintained equal among the cells amount per surface area, MOI, volume of overlay media, and virus inoculums. The titers obtained from these infections reflected a size dependant relationship, the larger the size the lower the titer.

Evaluation of concentration effect due to evaporation as a suspected factor revealed a similar relationship, the larger the size the lower the concentration effect, hence supporting the responsible factor. Indeed, there was 46.7% media loss in well of 24-well plate compared to only 0.8% in T150 flask. As a result, the titer of T150 flask was 35.3% of that of 24-well. The variation was minor when the titers were corrected for the lost

media. Such finding is explained by the fact that evaporation rate is correlated with the perimeter of the vessel where the evaporation occurs rather than to the surface area.

When it comes to large-scale production of viruses, it would be convenient for the manufacturers to use the largest available vessel instead of multiple smaller ones. Such strategy would result in lesser time and labor for maintenance of the infection vessels. It is not likely that it would be practical for manufacturers to translate the finding that for example 24-well plate yields higher titer than T150 flask into their production strategy for profitable industry unless some sort of high throughput automation had become feasible to employ.

4.5 Media pH

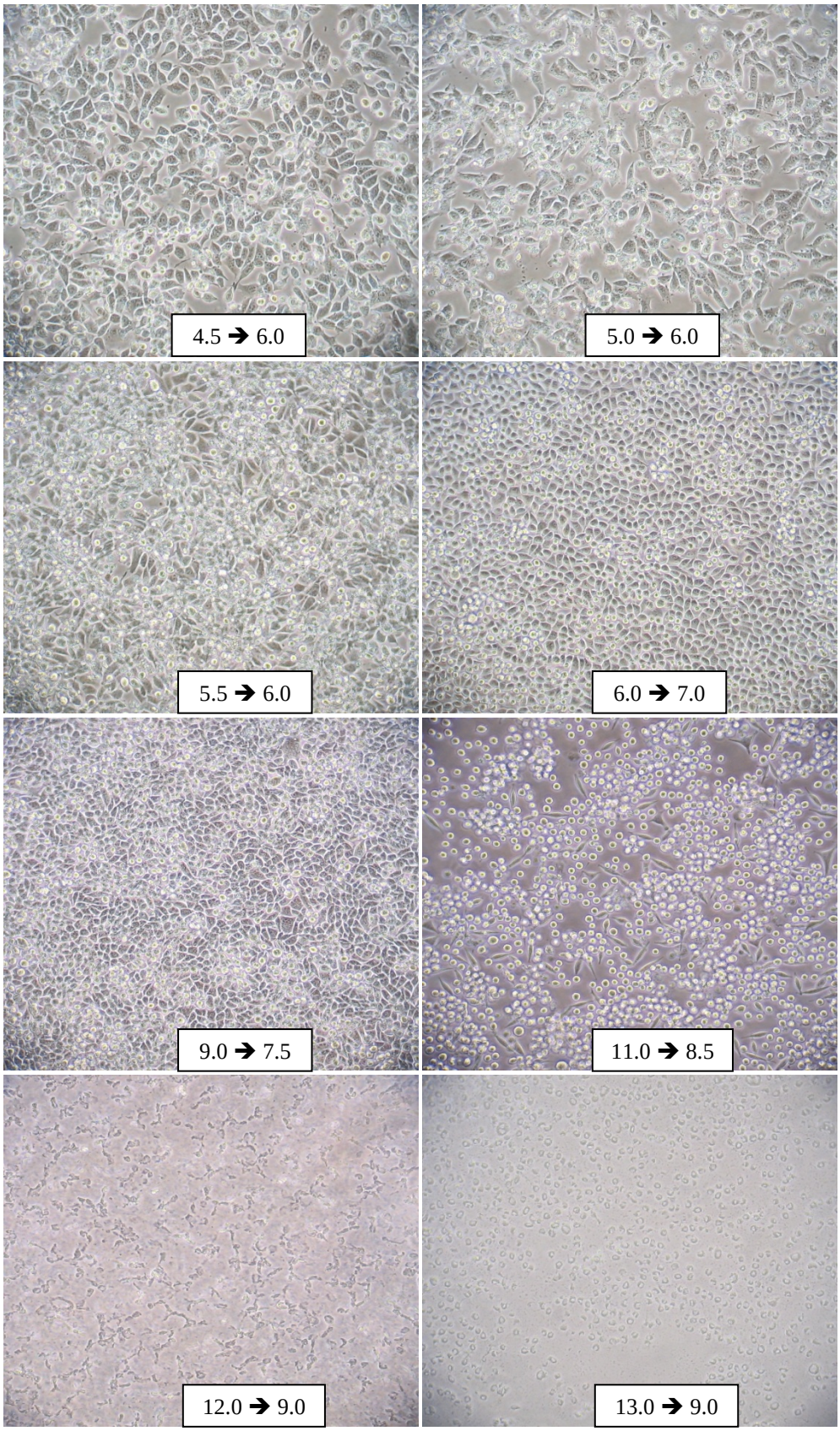
It is known that the right volume of the media overlay, the CO2 in the incubator, and the buffering ingredients are involved in the buffering capacity of the media. pH drift should be controlled in narrow range for the optimum condition that is best tolerated by the cells and the virus (Barngrover, Thomas et al. 1985; Nahapetian, Thomas et al. 1986). The optimum pH for mammalian cell culture is 7.2 to 7.4 but the metabolic processes would gradually alter the initial pH of the overlay media toward acidic as the incubation extended (Freshney 2000; Butler 2004; Phelan 2007). The buffering system in the media used narrowed and maintained the starting pH range of 4.5-13 to 6.5-9.0.

The results here showed that pH of the media overlay largely affect the virus production. One point difference in the starting pH as in 11 and 12 (corresponds to half point difference in the buffered pH of 8.5 and 9.0 respectively) resulted remarkably in as many as 5 $\log_{10}$ difference in reovirus T3D titers in L929 cells. However, the titer difference was moderate and gradual in the acidic media, the difference did not exceed 2

$\log_{10}$ between starting pH of 4.5-5.5 and 6.0 (corresponds to half point difference in the buffered pH of 6.5 and 7.0 respectively). Such observation might be attributed mainly to the cells as demonstrated by cell count, viability, and microscopic examination under the tested range of pH. Cells at starting pH 12 were entirely smeared compared to pH 11 which had a mixture of differentiating and rounding up populations. On the other hand, cells at starting pH 4.5, 5.0, and 5.5 were morphologically normal but less dense compared to that of pH 6.5 (**Figure 10**).

I believe that the disturbance in cell physiological activities (at least those related to virus infection support) due to suboptimal starting pH was reversed under restored optimal pH. However, at pH higher than 11 and lower than 4.5, the damage (shrinking due to hypertonicity of the highly acidic or basic media) was instant and beyond repair. Therefore, it could be concluded that L929 cells has a tolerable starting pH range of 4.5-11 (6.0-11 for maximum titers) and an optimum buffered pH range of 6.5-8.5 (7.0-8.5 for maximum titers).

a



b

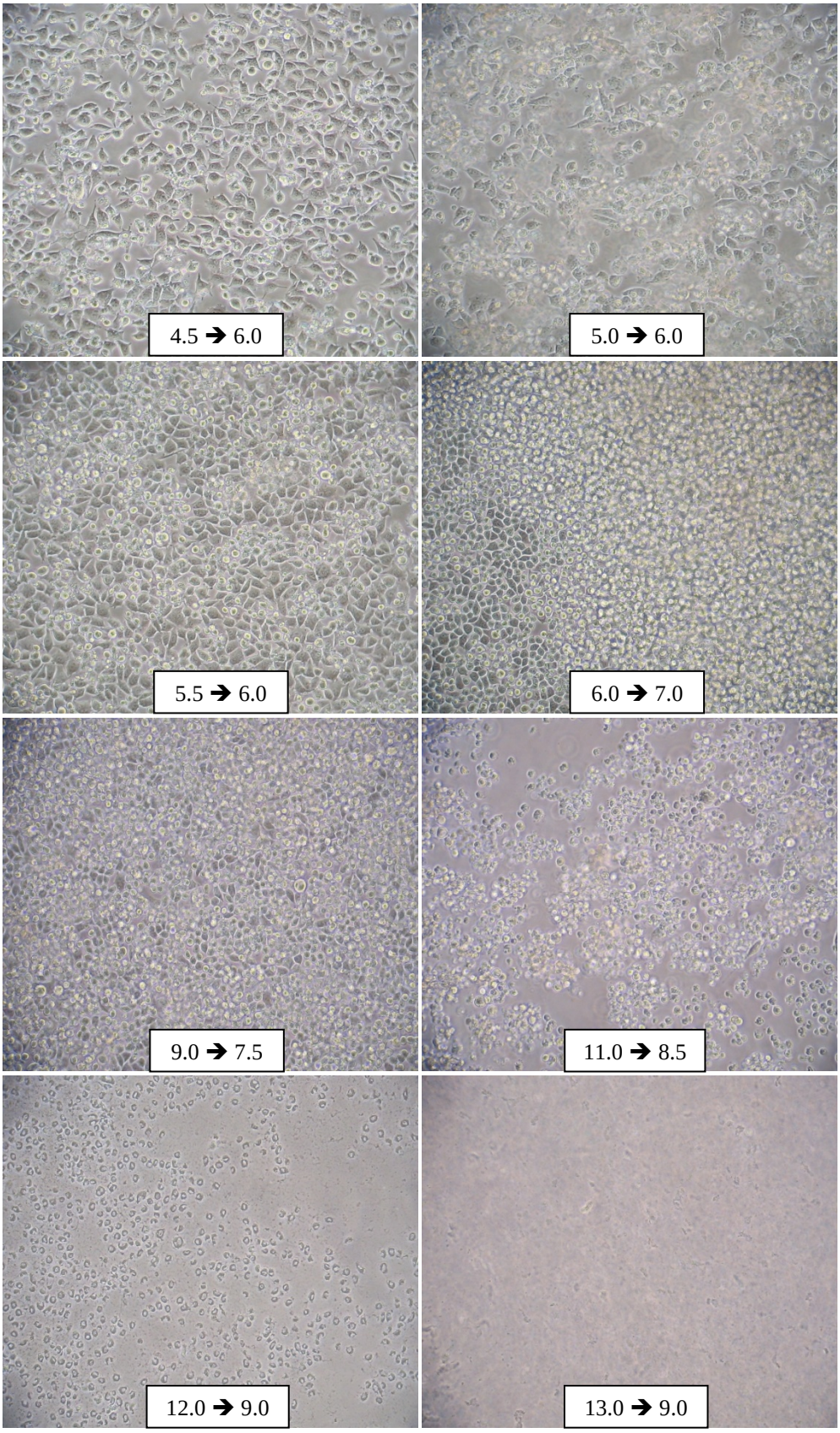


Figure 10. Morphological and proliferative disturbances of L929 cells due to the pH of the overlay media. L929 cells were seeded overnight in standard pH (7.2) in 24-well plates. Sub-confluent monolayers were then overlaid with 0.5 mL per 24-well of media with starting pH range of 4.5-13. Wells were photographed at 48 hrs (a) and 96 hrs (b). Starting pH at the left side of the arrow (→) and buffered pH at the right.

4.6 Future Directions

As the applications that employ viruses as natural killer for cancers or as therapeutic vector are ever increasing, future attempts to obtain massive amount of these agent may demand more complicated approaches such as genetically engineered cell lines or modified viruses. Such approaches may involve screening and characterization for propagation promoting genes or identification of factors that increase the cell threshold of virus replication. Moreover, such works may lead to elements in the viral genome that maximize the yield of fit progeny.

Microcarrier cell culture system employed in couple of studies showed promising results. Although the final titer was in the same range of that of stationary flasks, microcarrier systems provide a practical solution for large scale production of viruses from anchorage-dependant cells. The major advantage is more cells per surface area. Encouraging results obtained from production of influenza strain in microcarrier-based cell culture for the purpose of adopting the system for vaccine production. However, since there are extensive types of microcarriers, there have been some concerns that cells may behave differently in terms of attachment and differentiation in different microcarriers, which in turn may affect the infection process. Therefore, it should be taken into consideration the type of microcarrier employed and its effect on cells and virus titer.

The principle of metabolic processes modeling might constitute an option in the near future in the field of mass production of viruses. The massive amount of genomic and proteomic data in this era should encourage scientist to progress and explore how potentially useful outcomes might be obtained in this area.

4.7 Conclusion

This study has revealed couple of findings related to reovirus T3D production in L929 cell culture in which if they are considered for further studies would likely magnify the virus yield. Such outcome would be useful in mass virus production for research and therapeutic purposes. It was showed here that the condition that affects the capacity of L929 cell culture production of reovirus T3D was multifactorial with variable magnitudes. However, there could be difficulties in the appropriate interpretation of results obtained in such work. These are primarily due to the principles of adaptation and adjustment of cells in response to environmental alteration in one hand, and the fact that the maximum virus titer could be obtained in different combinations of altered conditions as showed on the other hand. Inferably, the rate of reovirus T3D replication in L929 cells and the time required for the maximum yield are varied over the course of changes in the growth condition. Further investigation in these aspects might reveal the specific conditions that promote the fastest rate of virus replication and the shortest time for maximum virus yield. Findings from such and further characterization works would likely positively impact the pharmaceutical production of oncolytic agent reovirus T3D in terms of efficiency and cost-effectiveness. Ultimately, such achievements in pharmaceutical industry would hopefully facilitate the affordability and hence, the conduction of mass pre- and clinical trials of reovirus-based cancer therapy.

5 References

ATCC (2010). "Product Information Sheet for ATCC CCL-1."

Barngrover, D., J. Thomas, et al. (1985). "High density mammalian cell growth in Leibovitz bicarbonate-free medium: effects of fructose and galactose on culture biochemistry." Journal of cell science **78**: 173-189.

Barta, V., W. T. Springer, et al. (1984). "A comparison of avian and mammalian cell cultures for the propagation of avian reovirus WVU 2937." Avian Dis **28**(1): 216-223.

Barta, V., W. T. Springer, et al. (1984). "A comparison of avian and mammalian cell cultures for the propagation of avian reovirus WVU 2937." Avian diseases **28**: 216-223.

Barton, E. S., J. C. Forrest, et al. (2001). "Junction adhesion molecule is a receptor for reovirus." Cell **104**(3): 441-451.

Bass, D. M., D. Bodkin, et al. (1990). "Intraluminal proteolytic activation plays an important role in replication of type 1 reovirus in the intestines of neonatal mice." Journal of virology **64**(4): 1830-1833.

Berard, A. and K. M. Coombs (2009). "Mammalian reoviruses: propagation, quantification, and storage." Curr Protoc Microbiol **Chapter 15**: Unit15C 11.

Berry, J. M., N. Barnabe, et al. (1999). "Production of reovirus type-1 and type-3 from Vero cells grown on solid and macroporous microcarriers." Biotechnol Bioeng **62**(1): 12-19.

Berry, J. M., N. Barnabé, et al. (1999). "Production of reovirus type-1 and type-3 from Vero cells grown on solid and macroporous microcarriers." Biotechnology and bioengineering **62**: 12-19.

Breitbach, C. J., T. Reid, et al. (2010). "Navigating the clinical development landscape for oncolytic viruses and other cancer therapeutics: No shortcuts on the road to approval." Cytokine & Growth Factor Reviews **21**(2-3): 85-89.

Burgener, A., K. Coombs, et al. (2006). "Intracellular ATP and total adenylate concentrations are critical predictors of reovirus productivity from Vero cells." Biotechnol Bioeng **94**(4): 667-679.

Burgener, A., K. Coombs, et al. (2006). "Intracellular ATP and total adenylate concentrations are critical predictors of reovirus productivity from Vero cells." Biotechnology and bioengineering **94**: 667-679.

Butler, M. (2004). Animal Cell Culture & Technology.

Butler, M. (2004). Animal cell culture and technology. London ; New York, BIOS Scientific Publishers.

Butler, M., A. Burgener, et al. (2000). "Application of a serum-free medium for the growth of Vero cells and the production of reovirus." Biotechnol Prog **16**(5): 854-858.

Carrel, A. (1912). "Pure Cultures of Cells." J Exp Med **16**(2): 165-168.

Castilho, L. d. R. (2008). Animal cell technology : from biopharmaceuticals to gene therapy. New York ; Abingdon England, Taylor & Francis Group.

Chandran, K., S. B. Walker, et al. (1999). "In vitro recoating of reovirus cores with baculovirus-expressed outer-capsid proteins mu1 and sigma3." Journal of virology **73**(5): 3941-3950.

Clarke, P. and K. L. Tyler (2008). Reoviruses: General Features. Encyclopedia of Virology. B. W. J. Mahy and M. H. V. v. Regenmortel. Oxford, Academic Press**:** 382-390.

Coffey, M. C., J. E. Strong, et al. (1998). "Reovirus therapy of tumors with activated Ras pathway." Science **282**(5392): 1332-1334.

Coombs, K. M. (1996). "Identification and characterization of a double-stranded RNA-reovirus temperature-sensitive mutant defective in minor core protein mu2." Journal of virology **70**(7): 4237-4245.

Coombs, K. M. (2006). "Reovirus structure and morphogenesis." Curr Top Microbiol Immunol **309**: 117-167.

Davis, J. J. and B. Fang (2005). "Oncolytic virotherapy for cancer treatment: challenges and solutions." J Gene Med **7**(11): 1380-1389.

Dryden, K. A., G. Wang, et al. (1993). "Early steps in reovirus infection are associated with dramatic changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction." The Journal of cell biology **122**(5): 1023-1041.

Dulbecco, R. (1952). "Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus." Proc Natl Acad Sci U S A **38**(8): 747-752.

Eagle, H. (1955). "Nutrition needs of mammalian cells in tissue culture." Science **122**(3168): 501-514.

Enders, J. F. and T. C. Peebles (1954). "Propagation in tissue cultures of cytopathogenic agents from patients with measles." Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine **86**(2): 277-286.

Enders, J. F., T. H. Weller, et al. (1949). "Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues." Science **109**(2822): 85-87.

Estes, M. K., G. Kang, et al. (2001). "Pathogenesis of rotavirus gastroenteritis." Novartis Foundation symposium **238**: 82-96; discussion 96-100.

Faisst, S. (1999). PROPAGATION OF VIRUSES | Animal. Encyclopedia of Virology. G. Allan and G. W. Robert. Oxford, Elsevier**:** 1408-1413.

Fields, B. N., D. M. Knipe, et al. (2007). Fields virology. Philadelphia, Wolters Kluwer Health/Lippincott Williams & Wilkins.

Fischer, A. (1924). "The Differentiation and Keratinization of Epithelium in Vitro." J Exp Med **39**(4): 585-588.

Freshney, R. (2000). Culture of Animal Cells A Manual of Basic Technique.

Freshney, R. I. (2005). Culture of animal cells : a manual of basic technique. Hoboken, N.J., Wiley-Liss.

Gaicu, N. (1987). "Outlook on the cell culture applications." Archives roumaines de pathologie experimentales et de microbiologie **46**(3): 195-209.

Garabedian, G. A. and L. V. Scott (1967). "Plaque assay for herpes simplex virus in L-929 (Earle) mouse fibroblasts." Proceedings of the Society for Experimental

Biology and Medicine. Society for Experimental Biology and Medicine **126**(2): 568-571.

Gey, G. O. and F. B. Bang (1951). "Viruses and cells: a study in tissue culture applications. I. Cells involved; availability and susceptibility." Trans N Y Acad Sci **14**(1): 15-24.

Gillian, A. L. and M. L. Nibert (1998). "Amino terminus of reovirus nonstructural protein sigma NS is important for ssRNA binding and nucleoprotein complex formation." Virology **240**(1): 1-11.

Grande, A. and J. Benavente (2000). "Optimal conditions for the growth, purification and storage of the avian reovirus S1133." Journal of virological methods **85**: 43-54.

Gstraunthaler, G., T. Seppi, et al. (1999). "Impact of culture conditions, culture media volumes, and glucose content on metabolic properties of renal epithelial cell cultures. Are renal cells in tissue culture hypoxic?" Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology **9**: 150-172.

Ham, R. G. (1963). "Albumin replacement by fatty acids in clonal growth of mammalian cells." Science **140**: 802-803.

Ham, R. G. (1965). "Clonal Growth of Mammalian Cells in a Chemically Defined, Synthetic Medium." Proc Natl Acad Sci U S A **53**: 288-293.

Hruby, D. E., D. L. Lynn, et al. (1980). "Cellular differences in the molecular mechanisms of vaccinia virus host range restriction." The Journal of general virology **47**(2): 485-488.

Joklik, W. K. (1999). REOVIRUSES (REOVIRIDAE) | Molecular Biology. Encyclopedia of Virology. G. Allan and G. W. Robert. Oxford, Elsevier**:** 1464-1471.

Jung, S., L. A. Behie, et al. (2004). "Optimization of reovirus production from mouse L-929 cells in suspension culture." Biotechnology and bioengineering **85**(7): 750-760.

Jung, S., L. A. Behie, et al. (2004). "Optimization of reovirus production from mouse L-929 cells in suspension culture." Biotechnology and bioengineering **85**: 750-760.

Kapikian, A. Z. (2001). "A rotavirus vaccine for prevention of severe diarrhoea of infants and young children: development, utilization and withdrawal." Novartis Foundation symposium **238**: 153-171; discussion 171-159.

Klein-Szanto, A. J., M. Terzaghi, et al. (1982). "Propagation of normal human epithelial cell populations using an in vivo culture system. Description and applications." The American journal of pathology **108**(2): 231-239.

Kuchler, R. J. (1974). Animal cell culture and virology. Stroudsburg, Pa.,, Dowden.

Kuchler, R. J. (1977). Biochemical methods in cell culture and virology. Stroudsburg, Pa.

New York, Dowden

exclusive distributor, Halsted Press.

Liemann, S., K. Chandran, et al. (2002). "Structure of the reovirus membrane-penetration protein, Mu1, in a complex with is protector protein, Sigma3." Cell **108**(2): 283-295.

Lockart, R. Z., Jr. and H. Eagle (1959). "Requirements for growth of single human cells." Science **129**(3344): 252-254.

MacDonald, C. (1990). "Development of new cell lines for animal cell biotechnology." Crit Rev Biotechnol **10**(2): 155-178.

Masters, J. R. W. (2000). Animal cell culture : a practical approach. Oxford ; New York, Oxford University Press.

Morgan, E. M. and H. J. Zweerink (1975). "Characterization of transcriptase and replicase particles isolated from reovirus-infected cells." Virology **68**(2): 455-466.

Morton, H. J., J. F. Morgan, et al. (1950). "Nutrition of animal cells in tissue culture; use of tweens in synthetic feeding mixtures." Proc Soc Exp Biol Med **74**(1): 22-26.

Nahapetian, A. T., J. N. Thomas, et al. (1986). "Optimization of environment for high density Vero cell culture: effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites." Journal of cell science **81**: 65-103.

Odegard, A. L., K. Chandran, et al. (2004). "Putative autocleavage of outer capsid protein micro1, allowing release of myristoylated peptide micro1N during particle uncoating, is critical for cell entry by reovirus." Journal of virology **78**(16): 8732-8745.

Offit, P. A. (1994). "Rotaviruses: immunological determinants of protection against infection and disease." Advances in virus research **44**: 161-202.

Olland, A. M., J. Jane-Valbuena, et al. (2001). "Structure of the reovirus outer capsid and dsRNA-binding protein sigma3 at 1.8 A resolution." The EMBO journal **20**(5): 979-989.

Parker, R. C., G. M. Healy, et al. (1954). "Nutrition of animal cells in tissue culture. VII. Use of replicate cell cultures in the evaluation of synthetic media." Can J Biochem Physiol **32**(3): 306-318.

Phelan, M. C. (2007). "Basic techniques in mammalian cell tissue culture." Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.] **Chapter 1**: Unit 1.1.

Rapp, F. and J. L. Melnick (1964). "Applications of Tissue Culture Methods in the Virus Laboratory." Progress in medical virology. Fortschritte der medizinischen Virusforschung. Progres en virologie medicale **6**: 268-317.

Rous, P. and F. S. Jones (1916). "A Method for Obtaining Suspensions of Living Cells from the Fixed Tissues, and for the Plating out of Individual Cells." J Exp Med **23**(4): 549-555.

Roy, P. (1996). "Orbivirus structure and assembly." Virology **216**(1): 1-11.

Sabin, A. B. (1959). "Reoviruses. A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described." Science **130**: 1387-1389.

Sabin, A. B. (1965). "Oral poliovirus vaccine. History of its development and prospects for eradication of poliomyelitis." JAMA : the journal of the American Medical Association **194**(8): 872-876.

Salk, J. E., U. Krech, et al. (1954). "Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines." American journal of public health and the nation's health **44**(5): 563-570.

Sanford, K. K., W. R. Earle, et al. (1948). "The growth in vitro of single isolated tissue cells." J Natl Cancer Inst **9**(3): 229-246.

Society, A. C. (2010). Cancer Facts & Figures.

Spandidos, D. A., G. Krystal, et al. (1976). "Regulated transcription of the genomes of defective virions and temperature-sensitive mutants of reovirus." Journal of virology **18**(1): 7-19.

Spier, R. E. (1991). "Large-scale mammalian cell culture: methods, applications and products." Current opinion in biotechnology **2**(3): 375-379.

Stanford, M. M., J. C. Bell, et al. (2010). "Novel oncolytic viruses: Riding high on the next wave?" Cytokine & Growth Factor Reviews **21**(2-3): 177-183.

Strong, J. E., G. Leone, et al. (1991). "Biochemical and biophysical characterization of the reovirus cell attachment protein sigma 1: evidence that it is a homotrimer." Virology **184**(1): 23-32.

Thirukkumaran, C. and D. G. Morris (2009). "Oncolytic viral therapy using reovirus." Methods Mol Biol **542**: 607-634.

Tyler, K. L. (1999). REOVIRUSES (REOVIRIDAE) | General Features. Encyclopedia of Virology. G. Allan and G. W. Robert. Oxford, Elsevier**:** 1454-1464.

Tyler, K. L., P. Clarke, et al. (2001). "Reoviruses and the host cell." Trends in microbiology **9**(11): 560-564.

Vaha-Koskela, M. J., J. E. Heikkila, et al. (2007). "Oncolytic viruses in cancer therapy." Cancer Lett **254**(2): 178-216.

Waymouth, C. (1959). "Rapid proliferation of sublines of NCTC clone 929 (strain L) mouse cells in a simple chemically defined medium (MB 752/1)." J Natl Cancer Inst **22**(5): 1003-1017.

Yang, W. Q., D. L. Senger, et al. (2004). "Reovirus as an experimental therapeutic for brain and leptomeningeal metastases from breast cancer." Gene Therapy **11**(21): 1579-1589.

Youngner, J. S., E. N. Ward, et al. (1952). "Studies on poliomyelitis viruses in cultures of monkey testicular tissue. I. Propagation of virus in roller tubes." American journal of hygiene **55**(2): 291-300.

Appendix

Reagent recipes

Gel saline (for 1 liter, stored at 4c)

8.0 g NaCl

0.03 g CaCl2

0.17 g MgCl2.6H2O

1.2 g H3BO3

0.05 g Na2B4O7.10H2O

3.0 g Gelatin, type A

PBS 1x (for 1 liter, stored at room temperature)

8.0 g NaCl

0.2 g KCl

0.15 g Na_2HPO_4

0.2 g KH_2PO_4

PBS-EDTA (for 1 liter, stored at room temperature)

8.0 g NaCl

0.2 g KCl

0.15 g Na_2HPO_4

0.2 g KH_2PO_4

0.2 g EDTA

Completed MEM (for 1 liter, stored at 4c)

940 mL 1x S-MEM (Joklik's S-MEM)

50 mL tissue-culture grade fetal bovine serum (FBS)

10 mL L-glutamine stock solution

Completed 2x 199 media (for 1 liter, stored at 4c)

900 mL 2x 199 medium (Medium 199 Modified)

60 mL fetal bovine serum (FBS)

20 mL L-glutamine stock solution

20 mL penicillin-streptomycin stock solution

Amphotericin B (1 mg/mL), 1000x (for 20 mL, stored at -20°C)

1 mL of 20,000x Amphotericin B solution stock solution

Completed to 20 mL with sterile ddH_2O

Amphotericin B (20 mg/mL), 20,000x (for 5 mL, stored at -20°C)

100 mg Amphotericin B solubilized powder, (Sigma, cat. no. A9528)

Dissolved in 5 mL sterile ddH_2O

Penicillin-streptomycin 100x (for 200 mL, stored at -20°C)

0.2 g penicillin G

2.0 g streptomycin sulfate

Completed to 200 mL with ddH_2O

2% Agar (for 250 mL, stored in room temperature)

5.0 g agar (BD, Bacto Agar, 214010)

Completed to 250 mL with ddH_2O

Autoclaved to sterilize and melt

Trypsin (25 mg/mL), 10x (for 50 mL, stored at -20°C)

1.25 g trypsin (1:250; porcine pancreas, Sigma)

0.425 g NaCl

Completed to 50 mL with 1x PBS