ANALYSIS OF CELL REORIENTATION AND PLAQUE MORPHOLOGY FOLLOWING HSV-1 INFECTION

KOKILA SHANKAR
SPRING 2017

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biology
with interdisciplinary honors in Biology and Biochemistry and Molecular Biology

Reviewed and approved* by the following:

Moriah Szpara
Assistant Professor of Biochemistry and Molecular Biology
Thesis Supervisor

Sarah Assmann
Waller Professor of Biology
Honors Adviser

Lorraine Santy
Associate Professor of Biochemistry and Molecular Biology
Honors Adviser

* Signatures are on file in the Schreyer Honors College.
ABSTRACT

Herpes simplex virus type 1 (HSV-1) affects a significant majority of people globally, causing oral and genital lesions, infectious keratitis, and encephalitis. As there is currently no cure, it is important to study the disease in vitro to identify mechanisms that may be potential targets for antiviral therapies. In HSV-1 research, productive infection is commonly modeled using Vero cells, derived from African green monkey kidney cells and lacking an interferon response, but their characteristics do not accurately reflect those of human cells. It is possible that different cell types have varied responses to viral infection. Consequently, it is critical to study HSV-1 infection across multiple cell types to understand all possible phenotypic outcomes following infection. Based on a study showing that infected cultured human keratinocytes (HaCaT cells) exhibited polarized reorientation and migration toward the site of infection (plaque) while Vero cells did not (Abaitua et. al, 2013), this project aimed to further study phenotypic variation in plaques following in vitro infection. To study these outcomes, I infected monolayers of both Vero and HaCaT cells with different strains of HSV-1 and monitored them over time through still and time-lapse imaging. I confirmed that HaCaT cells exhibit reorientation after infection unlike Vero cells. Additionally, between the two cell types, infection with different strains of HSV-1 produced multiple plaque morphologies. Plaque diversity was different in number and in shape and size. To further determine the role of HSV-1 in producing these variations, I examined the viral kinetics of these HSV-1 strains on both cell types. I conducted single step growth curve assays to see if the rate of infectious virus production in a single round of replication varied between the cell types. I observed that HaCaT cells had a slower rate of infectious virus production than in Vero cells. By conducting these analyses, we
can better determine the role that virus-cell interactions play in HSV-1 transmission and spread and hopefully elucidate through further study the mechanisms which cause these variations.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. iv

LIST OF TABLES ....................................................................................................................... v

ACKNOWLEDGEMENTS ........................................................................................................... vi

Chapter 1 Introduction ............................................................................................................. 1

  Virus Structure ....................................................................................................................... 2
  HSV-1 Infection and Replication ......................................................................................... 3
  HSV-1 Latency ....................................................................................................................... 7
  Cells Types and Viral Infection ........................................................................................... 9
  Virus-Host Interactions ....................................................................................................... 14
  Hypothesis .......................................................................................................................... 16

Chapter 2 Materials and Methods ......................................................................................... 17

  Cells and Viruses ............................................................................................................... 17
  Infection Assays ............................................................................................................... 19
  Plaque Picking and Serial Passaging ................................................................................ 20
  Single Step Growth Curves .............................................................................................. 21
  Imaging ............................................................................................................................... 22

Chapter 3 Results ..................................................................................................................... 24

  Reorientation is seen in HaCaT cells following HSV-1 infection, but is not completely
  absent in Vero cells ........................................................................................................... 24
  Vero and HaCaT infections yield differences in plaque size and morphology ............... 28
  17\text{orig} and 17-1 can produce homogeneous plaque variants ....................................... 31
  Strain 17-1 has a faster rate of infectious virus production in Vero than in HaCaT cells 33

Chapter 4 Discussions and Conclusions ............................................................................. 36

  Viral Causes of Reorientation and Plaque Diversity ......................................................... 37
  Cellular Causes of Reorientation and Plaque Diversity .................................................... 39
  Future Work ....................................................................................................................... 41

Appendix A Quest for Murine Alphaherpesvirus ................................................................. 45

  Introduction ....................................................................................................................... 45
  Materials and Methods ..................................................................................................... 47
  Results ............................................................................................................................... 50
  Discussion ......................................................................................................................... 52

BIBLIOGRAPHY ................................................................................................................... 55
LIST OF FIGURES

Figure 1. Structure of HSV-1 virion. .................................................................3
Figure 2. Herpesvirus infection pathway. ...........................................................6
Figure 3. Herpesvirus establishment of latency. ...............................................8
Figure 4. Vero and HaCaT monolayers..............................................................11
Figure 5. Comparison of plaque variants.........................................................12
Figure 6. HaCaT reorientation. ......................................................................14
Figure 7. Cell reorientation is observed in HaCaT cells, but is not observed in Vero cells....25
Figure 8. Viral infection forms larger plaques in HaCaT cells than in Vero cells...........28
Figure 9. Comparison of syncytial plaque variants between Vero cells and HaCaT cells......30
Figure 10. Clonal variants exhibit homogeneous plaque formation. .....................32
Figure 11. Viral kinetics in Vero and HaCaT cells over single replication round.........34
Figure 12. Optimization of alphaherpesvirus-specific primer PCR assay...............52
LIST OF TABLES

Table 1. Quantification of Plaque Diversity .................................................................29

Table 2. List of alphaherpesviruses in consensus sequence...........................................50

Table 3. Summary of Wild Mouse Tissue Swab Screening..............................................51
ACKNOWLEDGEMENTS

I would like to primarily thank Dr. Moriah Szpara for serving as an excellent mentor during my tenure in the lab. Her passion for her work and thoroughness in conducting everything done by the lab has helped to shape me into a better scientist with a greater appreciation for all of the work that scientific research entails. I would also like to thank Mackenzie Shipley and Dr. Colleen Mangold for being extremely supportive mentors in and out of the lab. These three people are fantastic representations of women in science and have taught me a lot about how to become a fellow woman in STEM.

Additionally, I would like to thank all of the members of the Szpara lab for all of their guidance, advice, and support. This lab has provided a stimulating environment in which to conduct research, and I have truly been influenced by every lab member’s dedication to and persistence in producing quality science.

I would also like to thank Dr. Sarah Assmann and Dr. Lorraine Santy for their guidance and useful feedback throughout the thesis writing and editing process. I would especially like to thank Dr. Assmann for also being a wonderful honors adviser over the last four years.

I would like to thank the Eberly College of Science and the American Society for Microbiology Undergraduate Research Fellowship for providing me with funding to conduct my research, and I would also like to thank Penn State University for providing me with the laboratory space and equipment with which to conduct my research.

Lastly, I would like to thank my family and my friends for their tireless support. They are the ones who have inspired me to enter scientific research and pursue all of my goals. Without them, I know I would not be where I am today.
Chapter 1

Introduction

Herpesviruses (of the order *Herpesvirales*) are a diverse group of viruses, affecting a wide range of species, from corals to horses to people. There are three families that make up the order – *Herpesviridae, Alloherpesviridae,* and *Malacoherpesviridae* (Pellett and Roizman, 2013). Herpesviruses are host-specific, meaning that infectivity across host species does not commonly occur as seen with many zoonotic viruses. Instead, herpesviruses have evolved over time with their hosts, so many different strains of virus can be found in a single species. Herpes simplex virus type 1 (HSV-1), of the subfamily *Alphaherpesviridae* within the *Herpesviridae* family, is a highly prevalent virus that affects a significant majority of people worldwide. As of 2012, approximately two-thirds of the global population aged 15-49 years old was infected with HSV-1 (Looker et. al, 2015). Among those who are infected with HSV-1, the resulting symptoms and severity of the virus vary widely (Roizman et. al, 2013). HSV-1 typically causes chronic recurring orolabial lesions, but it has also been shown to cause genital infection, and it is the leading cause of infectious blindness due to keratitis in the United States (Looker et. al, 2015; Liesegang, 2001). Recently, a growing percentage of HSV-1 has been shown to cause genital lesions, a symptom normally associated with herpes simplex virus type 2 (HSV-2) (Looker et. al, 2015). The virus can also be transmitted during birth to newborns and in rare cases has been shown to cause encephalitis – a phenotype unique to HSV-1 compared to the other herpesviruses (Brown et. al, 2003; Whitley, 1990). Currently, there is no cure for the virus, and antiviral therapies on the market, like acyclovir, treat the productive, replicating stage of the viral life.
cycle and minimize viral outbreaks and shedding. Specifically, the mechanism of acyclovir involves targeting the viral DNA polymerase, inhibiting it to prevent viral DNA replication (Gnann et. al, 1983). However, people who choose to take these drugs unfortunately have to take them periodically throughout their life to prevent viral outbreaks and suppress chronic shedding. Studying the many levels of HSV-1 pathogenesis, such as virus-host interactions and latency and reactivation, is crucial for developing more effective prevention measures and treatment strategies for this disease.

**Virus Structure**

Herpes simplex virus is a double-stranded DNA (dsDNA) virus with an approximate genome size of 152 kilobase pairs (Roizman et. al, 2013). It is organized into the following sections: unique long (UL), unique short (US), latency associated transcripts (LAT), inverted repeat long and short sequences (IRL, IRS), and terminal repeat long and short sections (TRL and TRS) (Fig. 1B). Each of these sections is integral to the virus’s ability to survive and replicate in its host organism. The unique long and short regions encode viral proteins necessary for virion structure and function, while the inverted repeat sequences serve as regions containing additional promoter genes (Chou and Roizman, 1986). The LAT sequence is primarily expressed during HSV-1 latency where it gets processed into short introns. Within the virion, the viral DNA is packed in a liquid crystalline state inside of an icosahedral nucleocapsid, which protects the genetic material from undergoing degradation. A proteinaceous tegument layer surrounds the nucleocapsid and includes more than 20 proteins with varying functions. Each virion has an outer lipid envelope studded with glycoproteins, which play a role in virus-cell interactions, and
are crucial for viral entry and egress (Fig 1A). Significant inter-strain variation within HSV-1 can be caused by factors such as base substitutions affecting restriction endonuclease sites or variability in repeat sequence number (Roizman et. al, 2013).

**Figure 1. Structure of HSV-1 virion.** Top left image is an electron microscope image that shows the structure of the virus particle and specifically the capsid and viral envelope. Top right image shows the layers in greater detail. Bottom image shows the full HSV-1 genome, specifically distinguishing the unique long and unique short regions. Image reproduced from *Principles of Virology* (3rd edition), S.J. Flint, L.W. Enquist, V.R. Racaniello, A.M. Skalka, 2009.

**HSV-1 Infection and Replication**

The primary site of HSV-1 infection occurs at the epithelial cell layer or mucosal membrane surfaces. In order to initiate infection, the virus must attach to the host cell membrane and be taken up into the cell through receptor-mediated endocytosis (RME). Glycoproteins on
the virion envelope play a large role in mediating the virus’s ability to attach to the host cell surface receptors. Glycosaminoglycans on the cell surface bind to the virions via glycoprotein C (gC) and glycoprotein B (gB). Integrally, glycoprotein D (gD) is needed to bind to one of the three possible cell surface receptors: nectin, herpesvirus entry mediator protein (HVEM), or 3-O-sulfated heparan sulfate proteoglycan. Once this occurs, a series of conformational changes activates the glycoprotein H/glycoprotein L heterodimer, and ultimately gB, to cause fusion between the viral envelope and cell membrane. A resulting downstream increase in intracellular calcium ion signaling triggers RME of the virion (Roizman et. al, 2013). This whole process is extremely rapid, taking under an hour for the virus to attach and penetrate the membrane (McClain and Fuller, 1993). Once enveloped, capsids are transported to the nuclear pores via retrograde transport by the microtubule motor dynein on the microtubule network. At the nuclear membrane, the viral DNA is released from the capsid into the nucleus and initiates gene replication and transcription using both its own machinery and the host’s (Roizman et. al, 2013).

Transcription occurs in a highly regulated cascade manner, in which specific genes are expressed at certain times. The first cycle includes five immediate-early genes (0-3 hours post infection (hpi)), transcribed in the absence of de novo viral protein synthesis. These act to inhibit the host antiviral response and to stimulate the transcription of early genes (4-6 hpi), which generally encode replication enzymes and DNA binding proteins. The late genes (7-9 hpi) are transcribed after the viral DNA has been replicated, and these genes encode the structural proteins necessary for progeny virion assembly. Virions are assembled by filling a premade capsid with progeny DNA concatemers in a liquid crystalline state.
Once the viral DNA has been replicated and transcribed to express the necessary proteins to build the capsid and tegument layers to protect the DNA, the viral progeny need to be transported back into the cytosol to obtain their outer lipid bilayer envelope and eventually egress from the cell (Fig. 2). Current research details three different possible models of viral envelopment. One proposed theory, the dual envelopment model, includes the nucleocapsid and tegument initially getting enveloped into the inner nuclear membrane, de-enveloped as it fuses with the outer nuclear membrane, and then getting re-enveloped by a transport vesicle. The full virion then travels to the cell membrane to be exocytosed. The lumenal model proposes that the virion acquires its double envelope in two stages. As the capsid enters the nuclear membrane, it gets one layer of its envelope, and it gets a second envelope layer as it exits the membrane. From here, it travels as a fully formed virion to the cell membrane. The third possible model is the nuclear pore model. In this model, the capsid exits the nucleus through an enlarged nuclear pore and remains unenveloped until it gets taken up by a cytoplasmic vesicle, which engulfs the capsid to form the double layer of the virion envelope (Roizman et. al, 2013). Viral proteins gB, gK, and UL20 play an important role in regulating fusion of the exocytotic vesicle with the cell membrane (Mettenleiter, 2002). Once a large number of viral progeny are produced, they are all transported to the cell membrane and are released, causing cell lysis and therefore cell death.
Figure 2. Herpesvirus infection pathway. The picture above depicts the viral life cycle during the productive phase of infection. Specifically, this figure details entry of the virus (1-4), viral replication (7-8), and the gene transcription cascade (9-18) in which virus enters the cell, uses host machinery to replicate its DNA and express viral proteins, and then gain its envelope to leave the cell. Image taken from Principles of Virology (3rd edition), S.J. Flint, L.W. Enquist, V.R. Racaniello, A.M. Skalka, 2009.
**HSV-1 Latency**

Once progeny virions bud from the host cell but before they lyse their host cell, they can go on to infect other epithelial cells, or travel to sensory neurons where they can establish a latent infection. Research also suggests that the virus can establish latency without first undergoing productive infection (Steiner et. al, 1990). HSV-1 can primarily establish latency in trigeminal or dorsal root ganglia within the peripheral nervous system. To enter neurons, virus particles fuse at the axon termini, and from there they travel via retrograde transport to the cell soma and establish an episomal state in the nuclei. In this form, no detectable viral replication occurs. LAT transcripts, highly stable introns, are the only transcripts that are highly expressed. Additionally, epigenetic regulation causes modification of the genome: chromatin conformation changes from a euchromatin state during productive infection to a heterochromatin state during latency, due to histone modification that silence viral lytic genes. At times of stress, or when the host becomes immune-compromised, the virus is able to reactivate from latency. Unlike productive infection, there is no highly ordered gene expression cascade to dictate viral DNA transcription during synthesis. Viral DNA synthesis starts, thus stimulating viral gene production to form a small number of progeny virions. Research suggests that *in vitro*, viral protein 16 (VP16) is a necessary protein that stimulates reactivation, and *in vivo*, infected cell protein 0 (ICP0) is necessary (Roizman et. al, 2013). These virions travel by anterograde transport with the aid of microtubule motor kinesins back to the epithelial cell layer, causing re-infection, viral shedding and potential transmission to new hosts (Fig. 3). Due to the fact that current antivirals only target the productive stage of the viral life cycle, latent virus is able to remain within a person throughout their lifetime and cause persistent re-infection as well as be transmitted to new hosts.
Figure 3. Herpesvirus establishment of latency. A) The movement of virions through the axon to establish latency in the soma and then movement back to the axon termini during reactivation. B) The establishment of latency in which the virus enters a sensory neuron and gets transmitted to the nucleus. Here, the virus circularizes into an episome and only expresses LATs, and uses virion host shutoff factor (Vhs) to destabilize expressed host and viral RNA. Top image adapted from Wilson and Mohr, 2012; bottom image adapted from Principles of Virology, S.J. Flint, L.W. Enquist, V.R. Racaniello, A.M. Skalka, 2009.
Cells Types and Viral Infection

*In vitro* research serves as a fundamental stepping stone in virology, as looking at viral infection in cell culture provides important information on how viruses interact with cells. Stripped from extracellular or *in vivo* interactions with other tissues and regulatory factors, the cell can perform functions with fewer added influences or variables affecting the study. When studying herpesvirus pathogenesis, epithelial cells and fibroblast cells are the most commonly used *in vitro* models because they are directly involved in the productive infection pathway; additionally, they are easy to obtain in bulk and can be used to study many different strains of herpesviruses. Epithelial cells are surface or mucous cells which line hollow organs, glands, and the outer surface of the body (NIH, 2014); fibroblasts are part of the connective tissue and primarily secrete extracellular matrix (Albert et. al, 2014). In humans, epithelial cells can be found in most organs such as the kidney or liver or mucosal or wet surfaces such as the inside of the nose and genitals. These mucosal surfaces are the ones most vulnerable to HSV-1 infections.

Vero cells are one of the most commonly used epithelial cell lines in HSV-1 research. Derived from a line of African green monkey (*Cercopithecus aethiops*) kidney cells, these immortalized cells are efficient models as they do not possess an interferon response and are thus extremely susceptible to viral infection (Rhim et. al, 1969; Desmyter et. al, 1968). In culture, Vero cells are small and have a sharp, polygonal shape similar to human fibroblasts (Fig. 4A). They are extremely adherent and grow in culture quickly. Additionally, HSV-1 grows to high titers in Vero cells, which is advantageous for growing and maintaining virus stocks. Vero cells are an inexpensive and efficient model for viral infection; however, this model is not the most accurate representation of infection in humans because the lack of interferon response, among
other characteristics, is not analogous to what occurs in human epithelial cells. Thus, many human-derived cell lines are used as alternative or additional models for *in vitro* assays. A commonly used human-derived model is the MRC-5 line. This cell line is derived from fetal human lung fibroblast cells, and has the ability to elicit a competent immune response (Smith et. al, 2001).

A less commonly used cell line known as adult human immortalized keratinocyte (HaCaT) are also used in HSV-1 *in vitro* studies because they mimic many of the characteristics of human fibroblasts (Schoop et. al, 1999). HaCaT cells are similar in shape to human fibroblasts as well; however, they appear rounder and reach confluency later than Vero cells (Fig. 4B). Additionally, because they are derived from epidermal tissue, they can potentially provide more insight into the host-virus interactions which occur during initial infection and at transmission (Kaplan et. al, 2012). Specifically, this cell line was used in a study conducted by Abaitua et. al (2013) which will be further discussed below.
Figure 4. Vero and HaCaT monolayers. A) Vero cell monolayer imaged at 90% confluency. B) HaCaT cell monolayer imaged at 75% confluency. Both images were taken in brightfield channel at 10X on a Nikon Ti-E epifluorescence microscope.

During HSV-1 infection in vitro, progeny virions spread to adjacent cells through cell junctions in a radial manner after initial replication in the host cell. Once a cell is filled with a high concentration of virions, it begins to round up and eventually undergo necrosis. In the same radial manner that viral spread occurred, a circular region of cell death due to apoptosis forms known as a plaque. Specifically, this is known as a zone of cytopathic effect (CPE). Another common infection phenotype occurs when the cell membranes instead fuse together to form a giant multinucleated area of infection known as a syncytium. Following infection, both CPE plaques and syncytia plaques can be formed, and strains of virus can be generated to produce a homogeneous plaque type (Fig. 5). In vitro, it has been seen that syncytia can form less
frequently than CPE plaques (Parsons et al, 2015); however, *in vivo*, due to the extracellular factors involved at the epithelial cell layer, it is possible that these syncytia are more commonly seen as the lesions one associates with symptomatic HSV-1 infection (Blank et. al, 1951; Spear, 1993)

Figure 5. Comparison of plaque variants. Infection of Vero cells with various strains of HSV-1 shows significant inter-strain diversity. Plaque purification of wild type strains shows
segregation into homogeneously phenotyped plaques, all of which were initially present in infection with the wild type “original” strain. Figure depicts CPE plaques (small and large) as well as syncytial plaques. Image reproduced from Parsons, L.R., Tafuri, Y.R., Shreve, J.T., Bowen, C.D., Shipley, M.M., Enquist, L.W., and Szpara, M.L. (2015). Rapid genome assembly and comparison decode intrastrain variation in human alphaherpesviruses. mBio 6, e02213-14.

Another interesting infection phenotype that has been described is the reorientation of cells following infection. Many cell types are polarized, with an apical and basal membrane that distinguish the top and bottom of the cell, respectively. In some cases, once a cell has been infected, adjacent cells can repolarize laterally to appear to move away from or towards the site of infection. In a study done by Abaitua et. al (2013), HaCaT cells were shown to have a different phenotype following HSV-1 infection than Vero cells. Specifically, this work showed that cells adjacent to the site of infection showed repolarization and reorientation towards the site of infection as compared to Vero cells (Fig. 6). Unfortunately, other primary research has yet to verify or contradict these findings. This drastic difference in infection phenotype could have strong implications in both in vitro and in vivo research, especially if the field continues to use cell culture models that are not wholly analogous to human models. Because this phenotype is visible in one cell culture model and not others, it indicates potential differences in virus-cell interactions that may not be taken into consideration when conducting research. Only by determining the causes of these differences can appropriate models be used for research studies.

Virus-Host Interactions

An important component of HSV-1 infections are the interactions the virus has with the host cell. As stated earlier, one major avenue of interaction is viral entry into the host cell. Between cell types, the mechanism of viral entry can vary. Of the three cellular receptors mentioned that gD can bind to for viral entry – nectin, HVEM, and 3-O-sulfated heparan sulfate – different receptors are utilized by specific cell types. For example, HVEM is member of the tumor necrosis factor (TNF) receptor family, and it is commonly expressed on T lymphocytes (Roizman et. al, 2013). Nectin-1 is the essential receptor for viral entry into neurons (Richart et.
al, 2003), and it is expressed broadly across a wide variety of cell lines and used for HSV entry (Karasneh and Shukla, 2011). Additionally, differences in viral entry have been found between HSV-1 and HSV-2. One of the primary differences is that HSV-2 uses gB rather than gC to bind to 3-O-S heparan sulfate (Agelidis and Shukla, 2015). This could be due to an evolutionary divergence between the different anatomical sites that HSV-1 and HSV-2 typically infect.

Another important component involved in HSV-1 infection is the host system immune response. Invading virions have unique pathogen-associated molecular patterns (PAMPs) that are detected immediately by the host cell’s pathogen recognition receptors (PRRs); this triggers the innate immune response. The cell secretes α and β type I interferons (IFN), which modulate other cellular enzymes, promote expression of interferon-stimulated genes (ISGs), and activate cellular pathways such as the JAK-STAT pathway in order to introduce an antiviral state in the cell. Interferons also mediate many other primary immune responses, such as recruitment of macrophages, natural killer (NK) cells, and major histocompatibility complex class I and II (MHC I and II), as well as cytokine secretion and the development of local inflammation. NK cells act to lyse pathogen-infected cells before the virus can spread to adjacent cells (Roizman et. al, 2013). Macrophages are recruited to the site of infection and release immune cell mediators to control IFN α/β-dependent cellular toxicity. Antigen presentation through the maturation of dendritic cells (DCs) also leads to production of large quantities of IFN-α by plasmacytoid dendritic cells, further stimulating the antiviral response. A few weeks after infection, immunoglobulins are also detected in the region of infection (Roizman et. al, 2013). Following the innate immune response, the adaptive or recurrent immune response takes over. Here, HSV-1 specific CD8+ and CD4+ T-cells, which have been primed by DCs, are triggered by IFN-γ to aggregate at areas peripheral to the site of infection. In some cases, these T-cells have actually
been seen to infiltrate latently activated trigeminal ganglia (Liu et. al, 1996). Overall, the immune response to viral infection is complex, and accordingly, HSV has developed strategies for evading this response, often resulting in variation in disease frequency and severity that varies based on the interactions between the virus and host immune response. The two primary methods under which these strategies fall are viral suppression of virion degradation by host lysozymes and viral degradation of cellular stress response mRNA (Roizman et. al, 2013). By suppressing virion degradation and/or degrading the cellular response, HSV can remain in the host cell longer, thus enabling the virus to replicate and produce progeny virions.

**Hypothesis**

The balance between host and virus is unique and can result in a variety of infection phenotypes *in vitro*. Similar to previous research (Abaitua et. al, 2013), I hypothesize that infection of different cell types with a single HSV-1 strain will produce unique phenotypic outcomes, not only in the presence or absence of reorientation, but specifically variation in plaque size and morphology. Additionally, I also predict that infection of the same host cell type with different strains of HSV-1 will also yield varying phenotypes similar to what was seen in Parsons et. al (2015). Compared to African Green monkey kidney cells (Vero), I anticipate that the species-matched human epithelial cell line HaCaT will have a slower rate of infection and a more fusogenic phenotype in which the cells form syncytia, which I plan to assess through imaging and viral kinetics assays. Furthermore, I predict that these variations in phenotype between virus strain are caused by the virus, potentially due to its efficiency of replication and pathogenesis in the host cell.
Chapter 2

Materials and Methods

Cells and Viruses

Vero cells (American Type Culture Collection # CCL-81) were obtained from a low passage aliquot. Cell aliquots (1 mL) were thawed in 19 mL of growth medium in a T-75 flask. Growth medium consisted of Dulbecco’s Modified Eagle’s Medium (DMEM) (HyClone #SH30081.02) + 10% fetal bovine serum (FBS) (HyClone #SH30071.03) + 1% L-glutamine (L-glut) (HyClone #SH30034.01) + 1% penicillin/streptomycin (pen/strep) (Life Technologies #15140-122). Once cells in T-75 flask reached at least 90% confluency, the culture was passaged at a 1:10 dilution into a new flask. The flask was washed with 1X phosphate-buffered saline (PBS) (Hyclone #SH30256.02), and 2 mL 1X trypsin-EDTA (Life Technologies 15400-054) was added to lift cells off the bottom of the flask. The cell solution was centrifuged at 1000xg for 2 minutes, and the resulting pellet was resuspended in 10 mL fresh growth media. One-tenth of the cell resuspension was added to a new T-75 flask, and cultures were kept in the 37°C incubator for approximately 3-4 days or until the cells again reached 90% confluency. One milliliter aliquots of cells in 10% dimethylsulfoxide (DMSO) (ATCC 4-X) were made from 1 confluent T-150 flask, stored in -80°C overnight, and then transferred into liquid nitrogen for long-term storage.

HaCaT cells (AddexBio # T0020001) were maintained in DMEM + 10% FBS + L-glut + pen/strep + 2 µM sodium pyruvate (Hyclone #SH30239.01). Cell aliquots (1 mL) were thawed in 19 mL of growth medium in a T-75 flask. Once cells in a T-75 flask reached at least 90%
confluency, the culture was passaged at a 1:5 dilution into a new T-75 flask. The flask was washed with 1X PBS, and 3 mL 1X trypsin-EDTA was added to lift cells off the bottom of the flask. The cell solution was centrifuged at 1000xg for 2 minutes, and the resulting pellet was resuspended in 10 mL fresh growth media. One-fifth of the cell resuspension was added to a new T-75, and cultures were kept in the 37°C incubator for approximately 3-4 days or until flask reached 90% confluency. One milliliter aliquots of cells in 10% dimethylsulfoxide (DMSO) (ATCC 4-X) were made from 1 confluent T-150 flask, stored in -80°C overnight, and then transferred into liquid nitrogen for long-term storage.

The two HSV-1 virus strains used were 17_{orig} and 17-1, and they are completely unrelated to each other. 17_{orig} was a laboratory stock of HSV-1 strain 17 which has not been plaque-purified. 17-1 was a clinically isolated sample obtained from the Nancy Sawtell lab. Working stocks of these viral strains were made from lab-passaged master stocks by infecting one confluent T-150 flask of Vero cells with each virus at a low multiplicity of infection (MOI) of 0.01. Virus dilutions were made in a total of 3 mL of infection medium (DMEM + 2% FBS + L-glut + pen/strep) and allowed to adsorb at 37°C for 1 hour. The flask was rocked gently every 15 minutes to avoid the cell monolayer drying out. After 1 hour, viral inoculum was aspirated, and 25 mL of fresh infection media was added to the flask, which was then left to incubate for 72 hours post infection (hpi). After 72 hpi, the cell monolayer was lysed, and the lysed cell solution was then collected, given 20µM HEPES, and aliquotted into 1 mL aliquots. These aliquots were stored at -80°C until use. Before using a virus working stock, the aliquot was removed from -80°C and freeze-thawed 3 times by alternating placement into liquid nitrogen for 30 seconds and 37°C water bath until the sample thawed (approximately 3 minutes). Viral titer was calculated by diluting freeze-thawed virus to limiting dilution in infection medium. The limiting dilutions were
plated in duplicate in a total volume of 200 µL in each well of a 6-well plate seeded with Vero cells; the cells were seeded one day prior at a density of 4x10^5 cells/well. After a 1-hour infection, the viral inoculum was aspirated and the cell monolayers were overlaid with a semisolid media containing 1% methocel (2% methylcellulose in 2X DMEM with 10% FBS and antibiotics). The plates were left in the 37°C incubator for 72 hpi, after which the methocel was aspirated, and the wells were stained with 0.5% methylene blue in 50% methanol solution for at least 30 minutes. To de-stain the plates, the methylene blue dye was aspirated and then each individual well was gently rinsed with deionized water. From there, plaque number per well was counted and viral titer (in PFU/mL) was determined using the following calculation:

\[
\frac{\text{total # of countable plaques}}{\text{total vol. plated based on lowest dilution}} \times \text{reciprocal of lowest countable dilution}
\]

The viral titer of \(17_{\text{orig}}\) was 1.66x10^7 PFU/mL and the titer of 17-1 was 1.37x10^8 PFU/mL.

**Infection Assays**

Viral infections using HSV-1 strains \(17_{\text{orig}}\) and 17-1 were carried out in both Vero and HaCaT cells. Cells were plated in a 6-well plate 24 hours prior to infection at a density of 4x10^5 cells/well for Vero and 6x10^5 cells/well for HaCaT. On the day of the infection, each virus aliquot was removed from -80°C and thawed for 2 minutes in a 37°C water bath. Virus aliquots were sonicated at 80% amplitude for 10 seconds at 1 second on/off. From each aliquot, 100 µL of virus was serially diluted in 900 µL of infection media to a final dilution of 5x10^-6 PFU/mL.
Growth media was then aspirated from the 6-well plate and each well was washed with 2 mL 1X PBS. A total volume of 200 µL of the diluted virus was added to each well. The viral inoculum was kept on the cell monolayers for 1 hour at 37°C, while the plates were gently rocked every 15 minutes. Then, the infectious material was aspirated and each well was overlaid with 2 mL of 1% methocel (2% methylcellulose in 2X DMEM with 10% FBS and antibiotics), after which they were left at 37°C for 72 hpi. Following completion of the infection assay, the methocel was aspirated and each well was stained with approximately 1 mL of 0.5% methylene blue in 50% methanol for a minimum of 30 minutes. The methylene blue was then aspirated, and wells were gently rinsed with deionized water. Plates were imaged either before or after staining.

**Plaque Picking and Serial Passaging**

Following the infection protocol described above, Vero and HaCaT cells were infected with 17_{orig} and 17-1 at a low dilution (MOI of 0.01) to look at plaque diversity. Following 72 hpi, plaques from three representative categories were isolated. Plaques were identified as CPE small, CPE large, or syncytial (Parsons et. al, 2015). Each plaque was drawn up using a 20 µL micropipette and added to 300 mL infection media + 20 µM HEPES. Each sample was either stored at -80°C immediately or used to infect fresh monolayers of their respective cell type. Each 6-well plate contained a HaCaT or Vero monolayer (consistent with the cell type from which the virus sample came) infected in duplicate with a plaque pick from each category: CPE small, CPE large, or syncytial. The monolayers were infected for 1 hour, overlaid with semisolid media, and left in the 37°C incubator for 72 hpi. The plaque-picking process was repeated if a heterogeneous mixture of plaques was still present in any of the wells. If homogeneous plaque morphology was
achieved, a 5-10 µL sample of the purified plaque type was drawn up using a 20 µL micropipette and added to 300 mL infection media + 20 µM HEPES mL infection media, and stored at -80°C.

Single Step Growth Curves

Single-step growth curve (SSGC) assays were conducted to measure the rate of infectious virus production in the cell over the course of a single round of replication (Ellis and Delbruck, 1939). Vero and HaCaT cells were plated 24 hours prior to infection in 35-mm² dishes at a density of 4x10⁵ cells/dish (Vero) and 6x10⁵ cells/dish (HaCaT). Eight dishes of each cell type were prepared for each biological replicate. On the day of infection, 3 dishes were counted and averaged to get the mean cell density per dish to be able to calculate the necessary virus dilutions needed to obtain a MOI of 10. Each dish was then infected with HSV 17-1 at an MOI of 10 for 1 hour given the following calculation:

\[
\frac{\text{avg. cell count} \times 10}{\text{virus titer}} + (200 \mu L \text{ infection media} - mL \text{ of virus}) = 200 \mu L \text{ viral inoculum}
\]

After 1 hour incubation, viral inoculum was aspirated and each dish received 2 mL of fresh infection media for its respective cell type. The infected plates were left in the 37°C incubator and at 1, 6, 12, and 24 hpi, a single dish from each cell type was harvested during each biological replicate. Using a P1000 micropipette, the media (approximately 2 mL) was collected and centrifuged at 1000xg for 2 minutes. After centrifugation, the supernatant was transferred to a clean 2 mL Eppendorf tube and labeled as the cell-free virus sample. Approximately 500 µL of infection media was added to each plate, and the adherent cells were scraped into the media. This
cell-associated virus was then added to the cell pellet from the centrifugation step. Each dish per cell type had both a cell-free and a cell-associated sample collected at each time point. All samples were freeze-thawed according to the procedure for viral stocks above. These samples were then titered on monolayers of Vero and HaCaT cells according to the infection protocol above.

**Imaging**

All images were taken on a Nikon Ti-Eclipse inverted epifluorescence microscope with Nikon NIS-Elements AR imaging software. To assess potential reorientation of cells following HSV-1 infection, 6-well plates of Vero or HaCaT cells were prepared and infected according to the protocol above with 17<sub>orig</sub> and 17-1 at a low MOI of 0.01. Following the 1 hr infection protocol, plates were kept in a Tokai Hit miniature stage-top incubator set to 37°C and 5% CO<sub>2</sub> for the entirety of imaging. One region of interest was selected per well based on the identification of early plaque formation. These regions’ locations on the x- and y-axis were set and imaged for the duration of each biological replicate. Images were taken in the brightfield channel at 10X magnification every 30 minutes from 24 hpi to 40 hpi (a total of 16 hours/33 images). The above imaging assay was repeated with fresh plates of HaCaT cells under the same conditions every hour from 36 hpi to 72 hpi (a total of 36 hours/37 images). Reorientation was determined by analyzing the ring of cells immediately surrounding the site of infection. As the plaque grew during the imaging period, adjacent cells were observed for changes in morphology (rounding up and/or elongating) and apparent rate of migration towards or away from the plaque.
site (moving slowly or quickly, distance traveled). Images of these plates were also taken after staining at 72 hpi to observe plaque morphology across the plate.

Individual 6-well plates were imaged during viral infection at 4X and 10X magnifications to get still images of individual plaques and regions of each well. The overall content of each well was imaged by scanning the whole well and capturing regions of interest (ROIs) at 10X magnification to get a representation of the overall plaque content. These ROIs were then stitched together using NIS-Elements software to create large 7x7 tiled images of the whole well. From these images, plaque diversity was calculated. To calculate plaque diversity, regions of interest (ROIs) were drawn around countable plaques and the area of each ROI was calculated in square millimeters using NIS-Elements AR software. Countable plaques were defined as plaques that were not disturbing or overlapping other plaques on the plate. The plaque type (CPE large, CPE small, syncytial), number, and size of plaques were recorded for 10 total plates.
Chapter 3

Results

Reorientation is seen in HaCaT cells following HSV-1 infection, but is not completely absent in Vero cells

The study conducted by Abaitua et. al (2013) demonstrated that following a low MOI infection with HSV-1 strain 17, HaCaT cells adjacent to the site of infection reoriented themselves and appeared to migrate towards the forming plaque; however, the study showed that Vero cells following HSV-1 strain 17 infection under the same conditions did not exhibit this unique phenotype. This was demonstrated both through live cell imaging performed from 16-24 hpi and still imaging obtained at 60 hpi. In order to maintain comparable experimental conditions as similar as possible to the above study, HSV-1 \(17_{\text{orig}}\) was used at a low MOI to infect both Vero and HaCaT cells. Time lapse images were acquired from 24-40 hpi because wild type HSV-1 strains that were not fluorescently tagged were used, and therefore it was more difficult to identify plaque formation before 24 hpi. Still images were acquired at 72 hpi, similar to the previous study. Further time lapse images were taken from 36-72 hpi to further study the reorientation phenotype in HaCaT cells (data not shown). To expand upon this study, another strain of HSV-1, 17-1, was used for comparison in both HaCaT and Vero cells. Based upon the findings of the previous study, I hypothesized that HaCaT cells would exhibit a reorientation phenotype while Vero cells would not; thus, this prediction further contributed to the overall hypothesis of the project which stated that human-like epithelial cells would respond differently to HSV-1 infection than non-human derived epithelial cells.
Figure 7. Cell reorientation is observed in HaCaT cells, but is not observed in Vero cells. Following an infection of HaCaT and Vero cells with either HSV-1 strain 17\textsubscript{org} or 17-1 at an MOI of 0.01, time lapse images were taken from 24-40 hpi. Panels A-D and J-M show HaCaT cells adjacent to the center of the plaque to appear elongated and stretched towards the center, whereas cells at the center appear compressed. Panels E-I and N-Q show Vero cells to appear to round up at the center of the plaque, but adjacent cells do not show any apparent reorientation towards the plaque center. In Vero cells, although a slight aura surrounds the site of infections, cells do not exhibit reorientation as clearly as in HaCaT cells. Scale bars show 200 µm. All images were taken at 10X using the brightfield channel on a Nikon Ti-E epifluorescence microscope.

After infection with HSV-1 strain 17\textsubscript{org} at an MOI of 0.01, time lapse imaging from 24-40 hpi demonstrates that HaCaT cells do indeed migrate toward the site of infection; there is a
pronounced movement towards the plaque from adjacent cells (Fig. 7; time-lapse movie data can be found at https://scholarsphere.psu.edu/collections/rb68xb988). In contrast, Vero cells do not have a distinguished migratory response following HSV-1 infection under the same conditions. At 24 hpi, plaques were just beginning to form in both cell types. Typically in Vero cells, plaques display a zone of cytopathic effect (CPE) in which the middle of the plaque has been cleared as cells have rounded up after necrosis and lifted off the dish. While some HaCaT cells also showed this same phenotype, most plaques in HaCaT monolayers feature cells rounded up and clustered on top of one another in the center, depicting a mound of dead cells rather than a cleared CPE plaque.

During the imaging time period, HSV-1 infection appeared to radiate outwards from the center of the plaque in HaCaT cells and create a visible ring around the boundary of viral infection. In Vero cells, the rounding up that was observed appeared to follow a pattern of CPE caused by lysing (Fig. 7). While a similar boundary ring was visible in Vero cells, only the cells directly next to the infected cells flattened before rounding up. However, following infection, HaCaT cells appeared to migrate towards the infection site by 36 hpi. The boundary ring around the plaque was larger than in Vero cells, and HaCaT cells on the edge of the boundary initially appeared to elongate and then became squished into the plaque once close enough. Plaques formed in HaCaT cells were generally larger than those formed in Vero cells, and they also had a larger area of cells that underwent reorientation. This same phenotype was visible in still images captured in Fig. 8. Images were acquired at 24, 48, and 72 hpi, and it was clear that HaCaT cells adjacent to plaques reoriented and migrated, while Vero cells did not.

Following infection with strain 17-1 at an MOI of 0.01, HaCaT cells exhibited similar phenotypes as those that appeared following HSV-1 17_{orig} infection. A boundary ring around the
site of infection was observed, and within this boundary, cells appeared to elongate and then compress into the plaque as it grew in size over time. Compared to infection 17\textsubscript{orig}, there appeared to be a higher ratio of CPE-type plaques in which the central cells were more rounded and lifted off the dish by 48 hpi (Fig. 8). Interestingly, the phenotype following HSV-1 strain 17-1 infection in Vero cells was not comparable to the phenotype observed after infection with 17\textsubscript{orig}. The boundary area surrounding the plaque was larger and more pronounced following infection with 17-1. During the imaging period, Vero cells were observed traveling from the edge of the infection boundary towards the center of the plaque. However, they did not display the elongating and subsequent compression phenotype that was observed in HaCaT cells. These differences in 17-1 infection compared to 17\textsubscript{orig} infection were also visible in still images taken as early as 24 hpi.
Figure 8. Viral infection forms larger plaques in HaCaT cells than in Vero cells. Panels A, E, and I show Vero cells infected with HSV-1 17\textsubscript{orig}, while panels C, G, and K show HaCaT cells infected with the same virus at an MOI of 0.01. Panels B, F, J show Vero cells infected with HSV-1 strain 17-1, and panels D, H, L show HaCaT cells infected with HSV-1 strain 17-1 at an MOI of 0.01. Starting as early as 24 hpi (panels A-D) plaques in HaCaT cells are larger than in Vero cells and already appear to exhibit reorientation. By 72 hpi, Vero plaques infected with strain 17\textsubscript{orig} (I) are closer in size to HaCaT plaques; however, infection of Vero cells with strain 17-1 yields smaller plaques by 72 hpi (K). All images were acquired at 10X magnification. Scale bars show 100 µm.

Vero and HaCaT infections yield differences in plaque size and morphology

To further assess the plaque morphology following HSV-1 infection, still images were taken of HaCaT and Vero cell monolayers at 72 hpi. Following the same low MOI (dilution of 5\times10\textsuperscript{-6}) infection described above, we saw that HSV-1 17\textsubscript{orig} produced fewer plaques than strain 17-1. This phenotype was seen in both cell types. However, while strain 17-1 produced more
plaques than $17_{\text{orig}}$, the plaques were smaller. For CPE large plaques following 17-1 infection, the average plaque size was 0.99 mm$^2$ for Vero cells and 2.1 mm$^2$ for HaCaT cells, and for CPE small plaques, the average plaque size was 0.24 mm$^2$ and 0.78 mm$^2$ on Vero and HaCaT cells, respectively. Conversely, following infection with strain $17_{\text{orig}}$, average plaque size of CPE large plaques was 1.21 mm$^2$ and 2.96 mm$^2$, and average plaque size of CPE small plaques was 0.39 mm$^2$ and 1.35 mm$^2$ for Vero cells and HaCaT cells, respectively (Table 1).

### Table 1. Quantification of Plaque Diversity

<table>
<thead>
<tr>
<th>Assay Condition</th>
<th>Average plaques/well</th>
<th>Plaque Morphology</th>
<th>% of population</th>
<th>Average Size ($\text{mm}^2$)</th>
<th>Reorientation Present?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero + $17_{\text{orig}}$</td>
<td>100</td>
<td>Syncytial</td>
<td>5.3</td>
<td>3.92</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE small</td>
<td>51.3</td>
<td>0.39</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE large</td>
<td>43.4</td>
<td>1.21</td>
<td>no</td>
</tr>
<tr>
<td>Vero + 17-1</td>
<td>156</td>
<td>Syncytial</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE small</td>
<td>72.6</td>
<td>0.24</td>
<td>partial</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE large</td>
<td>27.4</td>
<td>0.99</td>
<td>partial</td>
</tr>
<tr>
<td>HaCaT + $17_{\text{orig}}$</td>
<td>47</td>
<td>Syncytial</td>
<td>2</td>
<td>2.22</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE small</td>
<td>37.9</td>
<td>1.35</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE large</td>
<td>60.1</td>
<td>2.96</td>
<td>yes</td>
</tr>
<tr>
<td>HaCaT + 17-1</td>
<td>76</td>
<td>syncytial</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE small</td>
<td>38.5</td>
<td>0.78</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPE large</td>
<td>61.5</td>
<td>2.1</td>
<td>yes</td>
</tr>
</tbody>
</table>

Values were determined based on 10 wells per assay condition. Plaque morphology was based on examination of wells after staining with 0.5% methylene blue in 50% methanol following 72 hpi. Plates were scanned under Nikon Ti-E epifluorescence microscope using the brightfield channel at 4X magnification. Average plaque size was calculated using (regions of interest) ROI area calculation on NIS-Elements analysis software.

Plaque morphology phenotypes were quantified based on the proportion of plaque phenotypes present on each plate. As observed by Parsons et al (2015), the plaques were categorized into CPE large, CPE small, and syncytial. When comparing across four assay
conditions – Vero cells infected with 17\textsubscript{orig}, Vero cells infected with 17-1, HaCaT cells infected with 17\textsubscript{orig}, and HaCaT cells infected with 17-1 – plaque morphology was found to be relatively consistent for each virus strain, more so with 17-1 than 17\textsubscript{orig}. Infection with 17-1 produced a higher proportion of CPE small plaques than CPE large plaques in both Vero and HaCaT cells. Following 17\textsubscript{orig} infection, Vero cells had a higher proportion of CPE small plaques, whereas the reverse was true for HaCaT cells (Table 1). Additionally, only strain 17\textsubscript{orig} produced syncytial plaques, whereas strain 17-1 produced no visible syncytia in HaCaT or Vero cells. The syncytial plaques that formed following infection with 17\textsubscript{orig} looked unique as well. In HaCaT cells, these syncytia appeared to clump together and are raised in the fusogenic center, rather than containing a greater amount of enlarged fused polykaryocytes (Fig. 9). Some of the syncytial plaques observed showed evidence of sectoring, a phenotype in which components of the plaques appear to be from different virions. These plaques then appear “mixed” in their infection phenotypes as seen in Fig. 9B (Brown and Ritchie, 1975).

**Figure 9. Comparison of syncytial plaque variants between Vero cells and HaCaT cells.** After infection with 17\textsubscript{orig} at an MOI of 0.01, both images show a syncytial, or fusogenic, plaque at 4X magnification. A) Typical syncytial plaque on Vero cells, where cells are fused together and become multinucleated. B) Syncytial plaque on Vero cells has rounded cells and fused center. This plaque shows an example of sectoring. C) Syncytial plaque on HaCaT cells looks more clumped and raised. This plaque also appears to be sectored. Images were acquired on Nikon Ti-E epifluorescence microscope at 10X magnification at 72hpi. Scale bars show 0.2 mm.
17\textsubscript{orig} and 17-1 can produce homogeneous plaque variants

To observe whether any of the noted plaque phenotypes were more dominant than the others, viral variants that produced homogeneous plaque phenotypes were isolated from plaques from the infection assays described above. Infecting cells with plaque-purified strains of HSV-1 could determine if homogeneity could be maintained, as well as whether plaque types such as syncytia that are normally expressed in low proportions, would be present at higher numbers without selective pressures or competition from other plaque types.

In order to obtain purified stocks of virus, plaques were taken from monolayers infected with strains 17\textsubscript{orig} and 17-1. Samples of each category – CPE large, CPE small, and syncytial – were isolated by serial passage until the infection was homogeneous (Fig. 10). Isolated variants were passaged on the same cell type from which they were isolated. This process resulted in CPE small and CPE large plaque variants of 17\textsubscript{orig} and 17-1 as well as syncytial variants of 17\textsubscript{orig} derived from viral infections in both Vero cells and HaCaT cells.

On average, each variant remained homogeneous after two successive passages, similar to the Parsons et. al (2015) study. Once plaque variants were purified, viral infections of both HaCaT and Vero cell monolayers using HSV-1 CPE large and syncytial variants yielded plaques of the same morphology, indicating a successful purification. Interestingly, variations observed within these categories were also well preserved. For example, syncytial plaques that were different sizes and had different proportions of fused cells on Vero cells were purified from infection of strain 17\textsubscript{orig}. Following successive passages, the plaques maintained their own unique characteristics (data not shown). However, some of the CPE small variants present in Vero cells did not remain homogeneous and instead, reverted to produce CPE large plaques, and repeated attempts at purification did not eliminate this phenotype. All three subcategories of viral
plaque variants produced a similar number of overall plaques compared to the wild type strains of each virus. There were many CPE large and small plaques produced, as expected due to their high prevalence in infected monolayers. In regards to the syncytial variants, there were more plaques present after infecting with purified variants than following a wild type infection, possibly suggesting that syncytial variants may get selected against by the other viral variants present within a viral population during HSV-1 infection.

Figure 10. Clonal variants exhibit homogeneous plaque formation. Plaque-purified variants were derived from HSV-1 infection at an MOI of 0.01 with each strain of virus (17_{orig} and 17-1) on each cell type (Vero and HaCaT). Images were acquired using a Nikon Ti-E epifluorescence microscope. The images on the left were acquired at 10X magnification prior to plaque picking, and the images on the right were stitched 8x8 images acquired at 10X magnification after 72 hours following infection with each plaque-picked isolate. Scale bar for stitched images shows 1 mm.
Strain 17-1 has a faster rate of infectious virus production in Vero than in HaCaT cells

To determine the role of viral kinetics in the difference in number of plaques observed across infection assay conditions, single step growth curve (SSGC) assays were conducted at an MOI of 10 in triplicate. Vero cells and HaCaT cells were infected with strain 17-1 because the plaque phenotypes present in the previous assays were more consistent between the cell types and no syncytia were present. Additionally, strain 17-1 had a higher titer than strain 17_orig, which allowed for less volume of viral stock required to obtain a high MOI. At various time points following infection, the amount of infectious virus produced in both the cell-associated samples (from cell lysate) and cell-free samples (from media in dish) from each cell type was measured by titering each sample on fresh monolayers of Vero cells (Fig. 11).

After averaging the data from all of the replicates, it was determined that between the two cell types, there was no observed difference in titer over a single round of viral replication. Initial titers fell between $1 \times 10^4$ and $1 \times 10^5$ PFU per $1 \times 10^5$ cells. Titers at 24 hpi were high (between $1 \times 10^7$ and $1 \times 10^8$ PFU per $1 \times 10^5$ cells) for the cell-associated samples as well as the HaCaT cell-free sample, but lower (only $2.15 \times 10^6$ per $1 \times 10^5$ cells) for cell-free virus in Vero cells (Fig. 11).

While the viral titers were similar between cell types, the rates of infectious virus production over time differed. Cell-associated virus in HaCaT cells increased steadily for the first 12 hours, while in Vero cells, there was a greater increase between 6-12 hpi. The reverse was true for the amount of cell-free virus released into the media: in HaCaT cells, the rate of infectious virus released increased significantly from 6-24 hpi, while the rate of increase was an order of magnitude smaller in Vero cells. The kinetics of total infectious virus production was similar to the cell-associated virus. In HaCaT cells, infectious virus production increased at a slow, consistent rate over the replication period, while infectious virus production in Vero cells
accumulated at a slowed rate initially, and then greatly increased from 6-12 hpi, after which it plateaued.

![Graph A: Cell-associated Virus](image)

![Graph B: Cell-free Virus](image)

![Graph C: Total Virus](image)

**Figure 11. Viral kinetics in Vero and HaCaT cells over single replication round.** Single step growth curves were performed on 35-mm\(^2\) dishes of Vero and HaCaT cells with HSV-1 strain 17-1 at an MOI of 10. A) Comparison of viral titer of cell-associated virus normalized for the cell density present in each cell type. Rate of infectious virus production in HaCaT cells appears to be lower than in Vero cells. B) Comparison of viral titer of cell-free virus normalized for the cell density present in each cell type. We see that the rate of infectious virus
release into the media in HaCaT appears to be greater than in Vero cells, and that HaCaT cells release approximately 10-fold more infectious virus by 24 hpi. C) Comparison of titer of total virus present. The overall rate of infectious virus production in HaCaT cells is similar to that of Vero cells and a relatively equivalent viral titer is achieved by 24 hpi.
Chapter 4
Discussions and Conclusions

The goal of this research was to test the hypothesis that HSV-1 infection causes unique in vitro phenotypes in human-derived versus non-human derived epithelial cells. By conducting viral infections and examining cell monolayers through various types of imaging, I concluded that HaCaT and Vero cells exhibit differences following infection with the same strain of HSV-1. HaCaT cells displayed a reorientation phenotype following infection with either strain 17\textsubscript{orig} or 17-1 in which they turn towards the site of initial infection, while Vero cells did not (Fig. 7). Strain 17\textsubscript{orig} produced plaques that were on average larger in both HaCaT and Vero cells than plaques formed following 17-1 infection (Fig. 8). We also saw that syncytia only formed following infection with 17\textsubscript{orig} in both cell types, whereas this phenomenon was not seen in infection with HSV-1 17-1. Furthermore, we found that the rate of viral replication of HSV-1 strain 17-1 was slower in HaCaT cells than in Vero cells over the course of a single round of replication (Fig. 11). These findings demonstrate that variation exists during HSV-1 pathogenesis, and these variations appear to be influenced by particular cell types as well as different viral strains. Consequently, these observations should be taken into consideration when evaluating in vitro models or viral strains in HSV-1 studies.

In the study done by Abaitua et. al (2013) in which the reorientation phenotype identified in HaCaT cells was first characterized, the researchers performed an assay to demonstrate that paracrine signaling played a role in mediating cell migration. They found that infected cells release a soluble mediator which stimulates cell migration. This is one possible mechanism of cell migration; however, future studies are needed to determine if this is true for other cell types.
as well. Due to the complex nature of virus-cell interactions during viral pathogenesis, there are many possible host- or pathogen-based factors that could play a role in causing the cell reorientation phenotype.

**Viral Causes of Reorientation and Plaque Diversity**

One of the main findings from this research was a difference in both the size and number of plaques produced from infection with HSV-1 17\textsubscript{orig} versus 17-1. We found that strain 17-1 produced more plaques than 17\textsubscript{orig}, and the plaques were on average smaller in size (Table 1). Furthermore, infection with 17-1 showed no evidence of syncytia formation, whereas infection with 17\textsubscript{orig} produced a small proportion of syncytial plaques. Based on these observations, it is plausible that viral factors mediating cell entry and/or cell-to-cell spread are involved in causing these differences between strains (Balan et. al, 1994). Viral glycoproteins play an important role in viral entry into the host cell, as well as in cell-to-cell spread. There are 10-20 different glycoproteins in HSV-1, a few of which are major players that regulate these specific processes.

The glycoprotein E/glycoprotein I (gE/gI) complex has been shown to promote viral spread between epithelial cells by regulating intercellular interactions (Johnson et. al, 2001). This complex has also been implicated in the acquisition of the virion envelope (Dingwell and Johnson, 1998; Farnsworth et. al, 2003). As seen in Table 1, plaque sizes varied between cell type and viral strains used. These differences could be due to mutations such as single nucleotide polymorphisms (SNPs) in the amino acid sequence of these glycoproteins or from alterations in other viral proteins such as the UL51 gene product or ICP34.5 that this complex interacts with at cell junctions or within the cell (Mao and Rosenthal, 2003; Roller et. al, 2013). Another
glycoprotein that could be causing the variation seen in this research is gB. Many studies have shown that gB is responsible for syncytia formation (Gage et. al, 1993). We show that 17\textsubscript{orig} produced some syncytial plaques, while 17-1 did not produce any (Table 1). Based upon these findings, alterations in the gB gene could be involved in the lack of syncytia formation in cells infected with 17-1. Interestingly, a study conducted by Wisner and Johnson (2004), demonstrated that both the gE/gI complex and gB redistribute from the trans-Golgi network to cell junctions late after infection; these findings suggest the need for both gE/gI and gB to be present to promote cell-to-cell spread. Studying glycoproteins that are involved in regulating viral entry and cell-to-cell spread may provide insight into the possible causes of in vitro variations in plaque size and morphology between cell types and strains of HSV-1.

Cells have two main emergency shutoff responses to prevent viral spreading, apoptosis and necrosis. HSV-1 has mechanisms to counteract both in which viral proteins block the pathways mediating cell shutdown. Another potential cause of the variation described here is from viral proteins that are associated with cell death. It has been shown that viral immediate-early genes \textit{ICP4, ICP27, and ICP24} can inhibit apoptosis, and ICP6 and ICP10 have been shown to attenuate necrosis (Yu and He, 2016). These viral proteins would be interesting candidates to study as necrosis plays a large role in viral plaque formation that forms due to the clearance of cells in the cell monolayer (Nguyen and Blaho, 2006). It could be that genetic changes in these viral proteins between strains of HSV-1 may lead to differences in plaque size and/or morphology following infection. If the function of these proteins is affected, the virus may become better or worse at mediating cell shutdown. This would result in a plaque containing either fewer or more cells, respectively.
Cellular Causes of Reorientation and Plaque Diversity

One of the main findings from this research was that the rate of HSV-1 strain 17-1 viral replication in Vero cells was greater than the rate of viral replication in HaCaT cells. This finding suggests that cell type specifically may play a role in HSV-1 infection. Not only do Vero cells and HaCaT cells come from different species, but they also were derived from different tissues. Consequently, structural and functional differences in membrane proteins and cellular functions might have arisen, leading to variation in interactions with the same virus. Additionally, it was observed that more cell-associated virus was present than cell-free virus over the replication period, but HaCaT cells had a higher release of infectious virus than Vero cells, contributing to the difference in viral kinetics within Vero and HaCaT cells (Fig. 10). It is possible that these differences are due to cell-specific factors such as those that mediate cell entry, or those that contribute to plaque formation by mediating cell-to-cell spread.

In order to enter the host cell, HSV-1 has to first bind with membrane receptors on the outside of the cell. There are a few different types of cell surface receptors, one of the key families being heparan sulfate proteoglycans (HSPG). There are two different subtypes of these proteoglycans: syndecans, which are transmembrane integral proteins, and glypicans, which are peripheral membrane proteins (Bernfield et. al, 1999). While both subtypes contain chains of heparan sulfate (HS), the proximity of the HS chains to the cell membrane can contribute to the kinetics of the ligand (Bernfield et. al, 1999). It is unclear which category the HSPGs on Vero cells and HaCaT cells fall into, but it is possible that if the HSPGs on each cell type are unique, then the same virus may infect these cell types differently. Since HSPGs mediate viral entry, these binding events contribute to how quickly the virus is able to attach to the host cell membrane and subsequently enter the host cell. In Fig. 11, we see that the rate of infectious virus
production of strain 17-1 was slower in HaCaT cells than in Vero cells. This could be due to delayed viral entry into the host cell. It could also be due to alterations in host cell proteins that are involved in viral egress, as differences could cause variations in plaque morphology such as CPE formation versus syncytial formation. These include vesicular trafficking proteins such as the Rab family (Stegen et. al, 2013). Differences in host protein composition between Vero cells and HaCaT cells could contribute to the phenotypic differences identified here following infection with HSV-1 strain 17-1.

The extracellular matrix (ECM) comprises various structural and functional compounds, including collagen, proteoglycans, and laminins, that vary in proportion within different tissues (Bosman and Stamenkovic, 2003). The composition of the ECM secreted by each cell type – Vero vs. HaCaT – could be unique, which may lead to the observed variations in phenotype following HSV-1 infection. HSPGs have been shown to interact with cytokines and other secreted signaling factors, and they have also been implicated in cell migration (Bosman and Stamenkovic, 2003; Kresse and Schonherr, 2001). The proportion of HSPGs in the ECM and their propensity to interact with different signaling factors that may vary between cell types could cause alterations in HSV-1 infection phenotypes. Laminins found in the ECM are another structural protein that may play a role in cell migration (Bosman and Stamenkovic, 2003). In a study done by Broutian et. al (2010), it was shown that different strains of human papilloma virus (HPV) have unique interactions with components of the ECM in HaCaT cells. This finding could potentially translate to HSV-1 and explain why, in this case, HSV-1 strains $17_{\text{orig}}$ and 17-1 expressed different phenotypes following infection.

One major difference between the two cell types used in this study is that Vero cells do not possess an interferon response, but HaCaT cells have been shown to produce IFN-α and IFN-
γ (Rhim et. al, 1969; Federici et. al, 2002; Maher et. al, 2008). IFN-α plays an important role in stimulating the host immune response to viral infection, while IFN-γ is important for controlling infection after the initial immune response. HaCaT cells also express Toll-like receptors (TLR) which are important immune signaling factors that recognize pathogens (Kollisch et. al, 2005). Variations in the presence of an immune response between HaCaT and Vero cells could contribute to their unique responses to HSV-1 infection.

It was observed that HaCaT cells adjacent to the site of infection appear to migrate towards the center of a plaque (Fig. 7). Interestingly, the fact that HaCaT cells have an immune-sensing system and yet have been shown to migrate towards the site of viral infection potentially indicate the presence of other factors besides the virus. The study conducted by Abaitua et. al (2013) found paracrine signaling factors present in HaCaT cells, which perform functions such as recruitment of local cells to a specific site or induction of specific types of responses in surrounding cells (Belleudi et. al, 2010; Seeger and Paller, 2015). These factors may possibly contribute to the cell reorientation and migration phenotypes observed. The fact that HaCaT cells have these factors that interact with HSV-1 is important to take into consideration when conducting in vitro studies, as these factors may affect the physical phenotypes that are generated by a virus following infection.

**Future Work**

To test possible viral causes of phenotypic variation, the next assay to conduct in this study is a single step growth curve assay looking at the viral replication of HSV-1 17orig. Strain 17-1 was chosen to be studied first in this research because of its greater uniformity and higher
titer. Looking at the rate of infectious virus production of strain $17_{\text{orig}}$ will be useful for comparing the titers to those of strain 17-1 and for validating the differences between these strains (Fig. 8). Another logical experiment moving forward is to compare the genome sequences of the HSV-1 strains used in this work as both have been sequenced by our lab (Bowen et. al, unpub.; Pandey et. al, unpub). As done in Parsons et. al (2015), studying the genetic variation of HSV-1 strains can provide insight into what genomic differences can translate to any in vitro phenotypes observed. Proteins of interest include the viral glycoproteins and host cell shutdown response proteins discussed above. An alternative method is to use reverse genetics and knock out specific genes we may be interested in studying that encode for any of these proteins. Once a mutant virus strain has been made, we can then infect Vero cells and HaCaT cells and again compare the infection phenotypes.

To test possible causes of reorientation and plaque diversity that could be caused by the host cell, assays could be conducted that look for the presence of specific cellular proteins during virus infection. The effect that modulation of certain protein content in cell cultures have on phenotypic variation could also be studied. Immunofluorescence assays would be useful to determine the cell membrane proteins that are present in each cell type and where they localize. Immunofluorescence is a broad assay that can include either live cell imaging or fixed cell imaging, thereby providing different approaches to studying cell-virus interactions. Proteins like laminin can be stained with fluorescent antibodies, and live-cell time-lapse images can be collected to indicate the location of these proteins and determine whether or not they are more concentrated in areas of apparent migration. To determine whether the same virus interacts uniquely with each cell type’s membrane receptors, a viral protein such as a glycoprotein and a cell receptor protein such as an HSPG could both be detected using fluorescent antibodies, and
imaging could be performed to look for co-localization. This may demonstrate if an interaction occurs, and if so, where it occurs and in what proportion. Determining the extracellular signaling factors present in both Vero cells and HaCaT cells may also shed light on potential causes of variation. Modulating the exogenous amount of a particular signaling factor, such as cytokines \textit{in vitro}, in the presence or absence of HSV-1 may provide insight on whether the reorientation phenotype will consequently vary from the results seen in Fig. 7. Mutation or loss-of-function experiments could also be used to modify cellular proteins \textit{in vitro} and study the resulting effects on phenotypic variation in each cell type.

The underlying mechanisms of phenotypic diversity are not well understood, however, this is an important topic because of how it can vary \textit{in vitro} infection. \textit{In vitro} models for studying HSV-1 infection are typically inexpensive, easy to obtain in bulk, and can provide a more simplified system than many of the \textit{in vivo} models. When studying a particular pathogen using model systems, it is important to be informed about what interactions may be present between the components that could affect outcomes of the study; this knowledge can be used to design and use the most efficient model systems available. Knowing what factors affect infection phenotypes \textit{in vitro} may be translated to \textit{in vivo} systems. Using \textit{in vitro} models that take into account how a particular cell type interacts with the virus strain or pathogen being studied can translate to more efficient HSV-1 research.

HSV-1 is of great importance to study because it is highly prevalent globally and its pathogenesis is extremely complex. Because HSV-1 currently has no cure, studying the viral infection pathway at many levels, from initial virus-host interactions to establishment of latency and reactivation, will provide greater insight into mechanisms of infection which can be targeted for therapies. A better understanding of the \textit{in vitro} models available to study HSV-1 may also
translate to more effective basic research being conducted. Expanding our knowledge of HSV-1 is essential for the efforts to develop improved preventative measures and treatment strategies for this disease.
Appendix A

Quest for Murine Alphaherpesvirus

Introduction

All three *Herpesvirinae* subfamilies have strains which infect humans and cause disease (Glycopedia, 2015). However, herpesviruses have also been found in almost all mammalian species, such as primates, livestock, and domesticated animals, as well as different species of birds, fish, and reptiles.

In HSV research, mouse models are commonly used to study the pathogenesis of the virus *in vivo*. Mice serve as a useful system because they have been extensively characterized physiologically, provide a simplified avenue for genetic manipulation, and have a short lifespan which allows for quick turnover from newborns to adults. Moreover, it has been shown that mice have shared characteristics with humans such as similar activation of the innate immune system, mice can provide a lot of insight into the mechanisms of pathogenesis that occur in people (Kollias et. al, 2014; Mester and Rouse, 1991). Current mouse models used in herpesvirus research are based on the inoculation of mice with human strains of HSV through various sights such as the cornea, ear, brain, or foot pad (Leib et. al, 1999; Balan et. al, 1994; Engel et. al, 1996).

Herpesviruses have evolved over time to be host-specific; therefore, introducing a virus into a foreign host does not always produce natural phenotypes (Webre et. al, 2012). For example, in mice, HSV-1 infects the central nervous system after initial infection, which is not a
common occurrence in humans (Kastrukoff et. al, 2012; Shivkumar et. al, 2013). Therefore, using mouse models as a proxy for HSV infection in humans may not provide an adequate prediction of disease symptoms or be representative of viral evolution in humans. There is also ongoing research investigating the creation of “humanized mice” which involve human stem cells transplants, or altering the gut microbiome of the mice (Berges and Tanner, 2014; Tao and Reese, 2017). However, these proposed mouse models still ultimately involve infection of a non-human host with HSV-1, a virus that has not evolved along closely with this organism. Discovering a strain of alphaherpesvirus that infects mice as the primary host could potentially lead to virus models with more accurate disease representations of HSV infection that may translate more directly to viral infection in humans.

Beta- and gamma-herpesviruses have been discovered naturally in mice (Teterina et. al, 2009; Virgin et. al, 1997), but an alphaherpesvirus has not yet been found. Novel herpesviruses in different animal species have been discovered through polymerase chain reaction (PCR) screening of organ homogenates from samples within a species, such as fruit bats (Sasaki et. al, 2014). The PCR primers amplified a region of the herpesvirus genome that is highly conserved across different species in order to increase the probability of obtaining positive results. Common target genes used for screening are the DNA polymerase-encoding gene \( U_{L30} \) and the gene encoding glycoprotein B (gB) (Chmielewicz et. al, 2000). Both of these genes are highly conserved across all three herpesvirus subfamilies and play an important role in viral entry and replication in the host cell (Knopf, 1998; Farnsworth et. al, 2007). Thus far, experimental approaches to detect novel alphaherpesviruses in mice have not been well-documented.

Performing PCR on a target gene or organism tissue lysate specific to alphaherpesviruses could increase the chances of detecting a novel virus specific to this subfamily. Characteristics
unique to alphaherpesviruses include a short reproductive cycle, rapid spread in cell culture, and establishment of latency in sensory ganglia (Roizmann et. al, 1992). These common characteristics are potentially indicative of some level of genetic conservation across the genomes of many alphaherpesviruses (McGeoch and Cook, 1994).

I hypothesize that using a two-pronged approach consisting of screening wild rodent samples by using plaque assays to assess for the presence of virus as well as performing PCR using a consensus sequence degenerate primer design will be more effective in detecting a novel alphaherpesvirus than either method alone. Screening multiple samples obtained from both oral and genital sites of wild rodents increases the chances of finding a novel virus in the *Simplexvirus* genus. Additionally, designing PCR primers that are based on a general consensus from many alphaherpesvirus genomes is likely to be more specific than pan-herpesvirus degenerate primers. These primers would then be used to conduct PCR with the same rodent swab samples used for the infection screening, thereby confirming or denying the results found during that process.

**Materials and Methods**

Wild rodent samples were acquired from Dr. Kurt Vandegrift, a research associate in Dr. Peter Hudson’s lab at Penn State University. Swabs were obtained by capturing wild rodents in field sites located in Pennsylvania and New York, swabbing oral and anal areas, and placing swabs in tubes containing 500 µL media. For each rodent sampled, there were paired oral and anal swabs. A high throughput protocol was developed for sample purification, and this protocol was carried out in an alternative fume hood which vented directly outside rather than recirculated
air in order to prevent airborne spread of unknown infectious diseases. This consisted of taking 350 µL from each of 6 swab tubes, pooling them together for a total of 2.1 mL, and then pushing the sample through an 0.8 µm syringe filter (BD Falcon) to remove contaminants such as fungi and bacteria. The filtered samples were again filtered almost completely through an 0.1 µm syringe filter (BD Falcon). The amount of sample which remained on the filter (approximately 500 µL) was taken up with a micropipette and transferred to a clean Eppendorf tube. This process was performed to concentrate the virus on the filter.

These concentrated samples were then used to infect 6-well plates of Vero cells, seeded the day prior to infection at a density of 4x10⁵ cells/well (see cell maintenance and seeding protocol in Materials and Methods, p. 20). The entire volume of one undiluted pooled sample was used to infect each well. The samples were left to adsorb at 37°C for 1 hour, with gentle rocking every 30 minutes instead of the usual 15 minutes to allow potential virus a greater chance to settle to the bottom of the well and interact directly with the cell monolayer. After the 1 hour infection period, the inocula were aspirated and each well was overlaid with 2 mL semisolid media containing 1% methocel (2% methylcellulose in 2X DMEM with 10% FBS and antibiotics). The plates were left to incubate at 37°C for 72 hours, checking every 24 hours for signs of infection. To check for signs of infection, plates were placed under a light microscope and each well was screened at 10X magnification. If there were no positive signs of infection, indicated by plaque formation or unhealthy-looking cell monolayer, plates were bleached to minimize risk of other infectious disease transmission and then discarded in biohazardous waste.

For the PCR-based screening approach, degenerate primers were designed based on a consensus sequence of mammalian alphaherpesvirus DNA polymerase catalytic subunit genes, known as the U₅₃₀ gene for many species. Eleven alphaherpesviruses were used to construct the
consensus sequence. The sequences were obtained from GenBank (Table 2), and FASTA files were downloaded and imported into Geneious software. Multiple sequence alignments were created using CLUSTALW, and regions of high similarity were manually identified. At these regions, forward and reverse primers were generated using Geneious and tested for validity using Primer3Plus. The forward primer sequence was 5’-GCGCGACTGGCTGGCNATGCG-3’ and the reverse primer was 5’GCCGATGTMGTSACGGTGGC-3’, and this amplified a 211-bp sequence in the gene from base 2475 to 2686 on the consensus sequence. To test the primers, a gradient PCR was performed to optimize annealing temperature using a negative control containing no template DNA and a positive control using HSV-1 template DNA. PCR master mix consisted of 0.5 µM of each primer, 1X Clontech Advantage 2 DNA polymerase mix, 1.2 M betaine (Sigma B0300), 2% DMSO, 250 µM dNTPs, 1X Qiagen 10X buffer, and deionized H2O. Genomic DNA (gDNA) HSV-1 strain F_{large} was used as the template. The PCR cycle included denaturation for 30 seconds at 95°C, annealing for 30 seconds at a range from 50-70°C, and elongation for 1 minute at 68°C for 30 cycles. The resulting PCR products as well as the 100 bp ladder were mixed in 1X loading dye and samples were run on a 2% agarose gel for 50 minutes at 100V.
Table 2. List of alphaherpesviruses in consensus sequence

<table>
<thead>
<tr>
<th>Species name</th>
<th>Abbreviation used</th>
<th>Accession ID</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine herpesvirus 1</td>
<td>BHV-1</td>
<td>1487384</td>
<td>NC_001847.1</td>
</tr>
<tr>
<td>Bovine herpesvirus 5</td>
<td>BHV-5</td>
<td>2700710</td>
<td>NC_005261.2</td>
</tr>
<tr>
<td>Equine herpesvirus 1 (ORF30)</td>
<td>EHV-1</td>
<td>1487570</td>
<td>NC_001491.2</td>
</tr>
<tr>
<td>Equine herpesvirus 4 (ORF30)</td>
<td>EHV-4</td>
<td>1487595</td>
<td>NC_001844.1</td>
</tr>
<tr>
<td>Felid herpesvirus 1</td>
<td>FHV-1</td>
<td>8658557</td>
<td>NC_013590.2</td>
</tr>
<tr>
<td>Fruit bat alphaherpesvirus 1</td>
<td>FBHV-1</td>
<td>19621673</td>
<td>NC_024306.1</td>
</tr>
<tr>
<td>Herpes simplex virus type 1</td>
<td>HSV-1</td>
<td>2703462</td>
<td>NC_001806.2</td>
</tr>
<tr>
<td>Herpes simplex virus type 2</td>
<td>HSV-2</td>
<td>1487316</td>
<td>NC_001798.2</td>
</tr>
<tr>
<td>Macacine herpesvirus 1</td>
<td>MHV-1</td>
<td>1487425</td>
<td>NC_004812.1</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>PRV</td>
<td>2952496</td>
<td>NC_006151.1</td>
</tr>
<tr>
<td>Varicella-zoster virus (ORF28)</td>
<td>VZV</td>
<td>1487712</td>
<td>NC_001348.1</td>
</tr>
</tbody>
</table>

Sequences were obtained from GenBank (https://www.ncbi.nlm.nih.gov/gene/). All sequences listed have the U_l30 gene name, except where noted in the table. Accession ID and location on the genome are provided for reference.

Results

Overall, 474 Peromyscus leucopus and Mus musculus samples were screened (approximately 53%) (Table 3). However, none of the samples screened showed any sign of viral infection. Monolayers had no visible plaque formation, and cells looked comparable to healthy monolayers. A modified infection protocol was also implemented, which significantly increased the incubation time by 48 hpi following overlay with semisolid media. This altered infection protocol also did not yield any positive samples.
Table 3. Summary of Wild Mouse Tissue Swab Screening

<table>
<thead>
<tr>
<th>Species Sampled</th>
<th>Number of Samples Screened</th>
<th>Total Number of Samples</th>
<th>Percent of Samples Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mus musculus</em></td>
<td>162</td>
<td>243</td>
<td>67%</td>
</tr>
<tr>
<td><em>Peromyscus leucopus</em></td>
<td>312</td>
<td>486</td>
<td>64%</td>
</tr>
</tbody>
</table>

Each sample represents an oral and an anal swab from the same animal. Values were determined by how many samples from a given species were opened and pooled for screening out of the total number of samples for that species.

The primers designed to amplify the U₄₃₀ region of the positive control HSV-1 strain *F*₉ large nucleocapsid DNA were successful (Fig. 12). Of the 8 PCR products obtained from running the gradient PCR, 4 produced DNA bands on the gel. The bands were around 200 bp, approximately the same length as the U₄₃₀ amplicon. The gradient PCR assay was successful in that it not only demonstrated proper amplification of the correct gene, but it also identified which annealing temperature was the most optimal for extensive amplification of the U₄₃₀ gene product. The assay results showed that the cooler the annealing temperature, the cleaner and larger the band of product DNA was. However, thin bands greater than 1 kb in size were also observed in two of the PCR product samples.
Figure 12. Optimization of alphaherpesvirus-specific primer PCR assay. PCR reactions using alphaherpesvirus-specific primers targeting a region of the U130 gene were performed to amplify HSV-1 strain F_{large} template DNA. Products were run and separated on a 2% agarose gel for 50 min at 100V. Bands are at approximately 200 bp, which is the expected size range of the PCR products. Gradient PCR was performed at the following annealing temperatures: the product in lane 2 had an annealing temperature of 58.4°C, lane 3 had an annealing temperature of 60.5°C, lane 4 had an annealing temperature of 62.4°C, and lane 5 had an annealing temperature of 65.9°C. Bands in lanes 4 and 5 show amplification of unknown DNA.

Discussion

Creating a two-pronged approach to search for novel alphaherpesvirus detection was partially successful, but as the project is still in its early stages, improvements can be made to the experimental design. With the infection-based screen, it is possible that the experimental design allowed too many chances for the virus to be lost. If these samples contain any virions, potentially only one virion could be in the entire sample if the rodent was not infected with
herpes, and if it was not shedding at the time of sample collection. Consequently, failure to use the entire sample may result in virus being left undiscovered. The filtration process used to condense the virus also could result in virus loss: in the step using the 0.1 µm filter; pushing the inoculum through the filter should trap the virus in the filter. However, using a micropipette to collect the material on the filter could result in virions getting left behind on the filter or stuck on the pipette tip. Finding ways to streamline the protocol could increase the chance of successful virion capture. Additionally, during the infection period, it is possible that not enough time was given for potential virus to incubate and replicate. Clinical samples of HSV-1 have been shown to take up to a week to ten days to infect cell culture (Singh et. al, 2005), and the samples used in this study were only kept on the monolayers for up to five days in order to minimize the risk of diseases like hantavirus. Allowing a longer incubation time could help select for virus growth.

In the primer design approach, the next challenge is to ensure that the primer set is alphaherpesvirus-specific. As strains of beta- and gammaherpesvirus that infect mice have already been discovered, the goal is to find a novel alphaherpesvirus whose natural host is the mouse. To test for alphaherpesvirus specificity, PCR should be performed using strains from all three herpesvirus subfamilies as template DNA. Successful amplification of only alphaherpesvirus template DNA would confirm the primers’ specificity. Generation of more primer sequences could then be used to screen the wild rodent tissue swab samples. As the primers have already been optimized for annealing temperature, the PCR protocol should allow for maximum amplification of target DNA. However, the U130 gene is highly conserved across all subfamilies of herpesviruses (Knopf, 1998; VanDevanter et. al, 1996); therefore, it may not be the best target for an alphaherpesvirus-specific primer set. Alternative genes such as US3, a
protein kinase, have been found to be more commonly conserved in alphaherpesviruses, and thus they could potentially serve as more specific targets (Roizman et. al, 2013).

Ultimately, a two-pronged approach similar to the one proposed here could be useful in finding novel strains of alphaherpesvirus which naturally infect mice. The approach detailed in this study is a low-risk, high-reward method in which success is dependent on the number of samples that can be screened. Mouse models are one of the main avenues used to study aspects of herpesvirus infection in vivo; therefore, discovering a virus that is host-specific to mice would benefit the field and may result in disease phenotypes that more accurately reflect a natural HSV-1 infection. Improved understanding of disease phenotypes could then be translated to human disease, with the goal of elucidating new mechanisms for treatment or prevention.
BIBLIOGRAPHY


World Health Organization (2015). Globally, an estimated two-thirds of the population under 50 are infected with herpes simplex virus type 1. Media centre.

Academic Vita of Kokila Shankar
kis5502@psu.edu

EDUCATION

Schreyer Honors College, The Pennsylvania State University Class of 2017
B.S., Eberly College of Science
Major: Biology (Neuroscience option)
Minor: Biochemistry & Molecular Biology, Psychology
Honors in Biology and Biochemistry and Molecular Biology

LABORATORY EXPERIENCE

Research Assistant, Penn State Department of Biochemistry and Molecular Biology May 2015-May 2017
• Worked under Dr. Moriah Szpara, Assistant Professor of Biochemistry and Molecular Biology
• Honors Thesis project: Analyzing cell orientation and morphology following HSV-1 infection
• Infected multiple cell types with HSV-1 strains and qualitatively and quantitatively analyzed variation in diversity and viral kinetics
  o Received funding from ASM Undergraduate Research Fellowship for summer work
  o Presented poster at ASM Microbe 2017, New Orleans, LA
  o Presented poster at 2016 Eberly Fall Undergraduate Research Exhibition
• Summer and fall 2015 project: A Quest for Murine Alphaherpesvirus
  o Presented poster at 2015 Eberly Fall Undergraduate Research Exhibition
• Conducted an infection-based screen and designed a DNA sequence detection method to find novel strains of alphaherpesvirus in wild mouse tissue samples

Externship, The Johns Hopkins Hospital Department of Neurosurgery March 2015
• Shadowed ECoS and SHC alum Dr. Timothy Witham
• Observed doctor-patient relationships in a clinical setting and patient surgeries in the hospital operating room

Research Assistant, Penn State Department of Biochemistry and Molecular Biology Sept 2013-Nov 2014
• Worked under Dr. Melissa Rolls, Director of the Penn State Center for Cellular Dynamics
• Project: Examining the role of specific kinase pathways in Drosophila axon regeneration
• Used Drosophila biology to manipulate gene lines and perform microscopic injury assays in vivo
• Analyzed results using confocal microscopy and quantitative image analysis

Research Intern, Penn State Hershey College of Medicine May 2014-Aug 2014
• Schreyer Honors College MD/PhD Summer Exposure Program
• Performed research in the Department of Pharmacology under Dr. Robert Levenson
• Summer project: Examining the Interaction Between Neuroligin-2 and α-synuclein
  o Presented poster at Summer Undergraduate Research Symposium
• Used protein-protein interaction assays and confocal microscopy image analysis to test novel interactor proteins in inhibitory synapses
• Shadowed in the Allergy, Asthma, and Immunology clinic under Dr. Faoud Ishmael

COMMUNITY SERVICE

• Haunted-U Fall 2013-2016
• EYH STEM Program Spring 2014, 2016, 2017
• Exploration-U Fall 2015
• Discovery-U Spring 2014

AWARDS AND HONORS

• American Society for Microbiology Undergraduate Research Fellowship 2016-2017
• Eberly College of Science Undergraduate Research Support Award 2015, 2016
- Eberly College of Science H. Jacob Hanchar Neuroscience Scholarship 2015
- Dean’s List Fall 2013-Spring 2017
- Provost Award – 2-year University-wide academic excellence scholarship Fall 2013-Spring 2015
- Schreyer Honors College Academic Excellence Award
- Braddock Scholarship

**ACTIVITIES AND LEADERSHIP ROLES**

- Learning Assistant, SC 297: Intro to Research in Science Fall 2016
- Schreyer Honors College SHO Time Orientation Mentor Fall 2014, 2016
- Tech Tutor, Penn State Information Technology Services May-August 2016
- Curriculum Mentor, Science-U July 2016
- Schreyer Honors College SHO Time Orientation Mentor Fall 2013-Spring 2017
  - Recruitment Chair 2014-2015
  - General member since Fall 2013
- Science LionPride Fall 2013-Spring 2017
  - Relay for Life Team Captain 2014
  - General member since Fall 2013
- Ballroom Dance Team Fall 2014-Spring 2017
  - Ballroom Manager and Instructor for Ballroom Dance Club 2016-2017
  - Teaching Assistant for the Advanced Ballroom class Spring 2016, 2017