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IN VITRO CHARACTERIZATION OF HERPES SIMPLEX VIRUS TYPE 1 CLINICAL ISOLATES WITH RESPECT TO THEIR POTENTIAL AS GENE VECTORS

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Herpes simplex virus 1 (HSV-1) is a common human pathogen that can be found in approximately half of the population. Typically, it causes relatively mild symptoms such as cold sores, though HSV-1 can also enter a latent stage and become undetectable to the immune system, which allows it to persist in an individual for their entire life. However, HSV-1 is also one of the most promising candidates for gene therapy vector development as it possesses multiple beneficial properties that set it aside from other potential oncolytic viruses. These include the capability for repeated dosing, a well-established neurovirulence gene that can be deactivated, and the capacity to support large transgenes. Accordingly, the only currently available oncolytic virus approved in the western world is based on HSV-1 with multiple other vector prospects currently in clinical development.

A set of 36 HSV-1 clinical strains isolated from patients was subjected to a panel of tests in order to determine their potential for further vector development. To this end, the strains were tested for multiple parameters, such as their replication characteristics, growth rates, oncolytic potential, and drug resistance. Several strains with promising results in view of oncolytic vector development could be identified, such as strains possessing significant oncolytic potential, high overall infectivity, or a significant tendency toward lateral spreading from cell to cell. While further testing will be required to make decisive conclusions, the results of this thesis serve as a useful baseline for future projects.

Key words: Herpes Simplex Virus, gene therapy, oncolytic vector, clinical isolate, antivirals

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1. Introduction

1.1 Herpes simplex virus

1.1.1 *Herpesviridae*

Herpesviridae are a large family of DNA viruses, consisting of 115 species within three subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Gatherer et al., 2021). The viruses in these subfamilies resemble each other in various ways: for example, they share similar replication characteristics, host ranges, and reproductive cycles (Davison, 2007). An important facet of all these viruses, however, is the fact that they are capable of forming a latent infection whereby only a small fraction of the viral genes are expressed (Davison, 2007). This state renders the virus nigh undetectable and can allow for the virus to persist for a lifetime (Roizman & Whitley, 2013; Sawtell & Thompson, 2021).

Table 1. Human herpesviruses and the diseases they cause.

Virus	Also known as	Typical pathology
HHV 1	Herpes simplex virus type 1	Orolabial herpes Genital herpes
HHV 2	Herpes simplex virus type 2	Genital herpes
HHV 3	Varicella-zoster virus	Chickenpox Shingles
HHV 4	Epstein-Barr virus	Infectious mononucleosis Various lymphoproliferative diseases (Burkitt's lymphoma)
HHV 5	Cytomegalovirus	Infectious congenital diseases
HHV 6a	Roselovirus	Unknown pathology
HHV 6b	Roselovirus	Sixth disease (exanthema subitum)
HHV 7	Roselovirus	Unknown pathology
HHV 8	Kaposi's sarcoma-associated herpesvirus	Kaposi's sarcoma

Though only nine members of *Herpesviridae* are known to primarily spread between humans, the viruses are quite distinct from each other even within this small group (Table 1). Varicella-zoster virus is known for causing chickenpox and shingles, Epstein–Barr virus has been implicated with infectious mononucleosis and various types of cancer, cytomegaloviruses are recognized as the most important cause of infectious congenital disease in the world, Human Herpesviruses (HHV) 6 and 7 are considered the causative agent of *exanthema subitum*, and HHV 8 is associated with Kaposi's sarcoma (Knipe &

Howley, 2013). However, arguably the most widely known members of this virus family are herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), also referred to as HHV 1 and 2, respectively (Roizman et al., 2013). Of these viruses, HSV-1 has garnered the largest amount of interest from the scientific community (Mody et al., 2020).

1.1.2 A brief history of HSV

Herpes simplex viruses have been accompanying humans for far longer than the scope of recorded history extends. Indeed, they are closely analogous to the herpesviruses of nonhuman apes, and interestingly, HSV-2 seems to have originally stemmed from a nonhuman host, as it is more closely related to the chimpanzee herpes simplex virus than HSV-1 (Wertheim et al., 2014). The first records of herpes-like symptoms are from Egypt and date as far back as 1500 BCE (Ebbell & Banov, 1937), though the virus gained its name in ancient Greece where the scholars of the time used the word “herpes”, which means to creep or to crawl, to describe a type of lesion spreading amongst the populace (Roizman & Whitley, 2001). Another anecdote from the 1st century alleges that herpes had grown so rampant in the Roman Empire that the reigning emperor, Tiberius, attempted to control the spread by banning kissing in all public ceremonies (Chodosh & Ung, 2020). Of course, the concept of a virus was completely foreign to the people of the ancient empires, and only in the late 19th century was it even definitively proven that herpes could spread from person to person (Vidal, 1873).

In the 20th century, medicinal research progressed by leaps and bounds, and the newly created field of virology was no exception. Multiple important discoveries regarding HSV were made, perhaps chief among them the fact that herpes was, in fact, caused by a virus (Löwenstein, 1919). Other milestones include the discovery of the fact that the virus targets the nervous system (Goodpasture, 1929), is capable of causing a latent infection (Burnet & Williams, 1939; Cushing, 1905) and that there were two separate types of the virus, HSV-1 and HSV-2 (Schneweis, 1962). Moreover, the development of plaque assays for virus quantification (Cooper, 1962) and other such methods helped pave way for our modern, more comprehensive understanding of the virus.

1.1.3 Epidemiology

Of the two herpes simplex viruses, HSV-1 is more common. It has a seroprevalence of around 66.6% of the global population, while HSV-2 can be detected in about 13.2% of

people (James et al., 2020). However, these estimates do vary depending on the region: for example, in Finland, the percentages resemble the global average, with HSV-1 having a seroprevalence of roughly 52% and HSV-2 13% (Pebody et al., 2004), but in the developing world HSV-2 appears to be much more ubiquitous, with up to 80% of the women of sub-Saharan Africa carrying the virus (Weiss, 2004). Classically, HSV-1 has been linked with cold sores whereas HSV-2 has been known as the primary pathogen leading to genital herpes (Gupta et al., 2007). However, in recent years this dynamic has started to shift, as HSV-1 accounts for more and more genital infections, especially among younger females in the developed world (Looker et al., 2015; Tuokko et al., 2014).

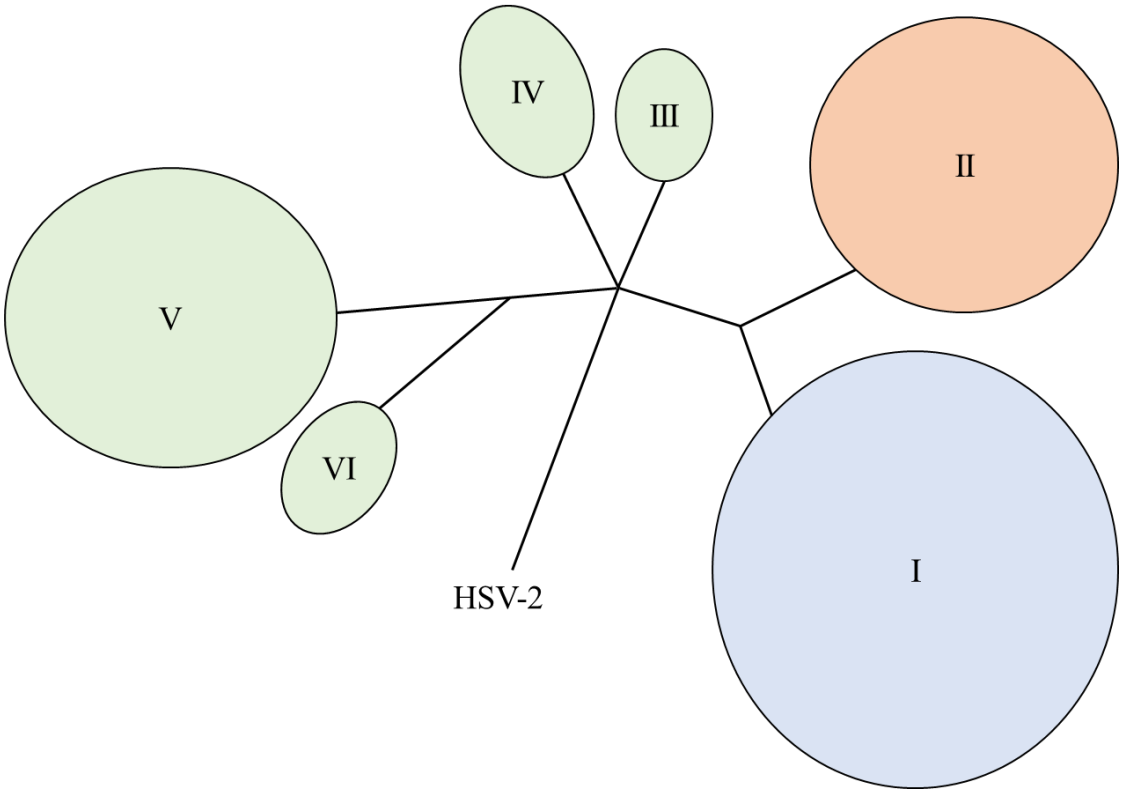

Figure 1. A simplified depiction of HSV-1's clades, based on the graph from Kolb et al. (2013). The virus' genealogy can be divided into six distinct clades. Clade I and clade II are sometimes referred to as "Western" and "Eastern" strains, respectively, while the rest of the strains form the "Southern" clade.

Indeed, as a result of its long coexistence with humans, the HSV genome appears to differ slightly depending on where it originates from (Bowen et al., 2019). By analyzing and comparing the genes of circulating HSV-strains from around the world, it is possible to create a phylogenetic tree for HSV-1, which features three distinct clades based on their origin (Kolb et al., 2011). These three clades, then, can be classified as the "Western", or North American/European clade, "Eastern", or Asian clade, and "Southern", or African

clade (Kolb et al., 2013). However, these variations are not geographically exclusive to any continent and can co-exist alongside each other due to factors such as immigration (Norberg et al., 2004). Genomic sequencing of Finnish isolates suggests that the Finnish circulating strains commonly fall into two of these clades, Western or Eastern (Bowen et al., 2019). It is important to note, however, that this three-clade structure might not be entirely accurate. The work that led to the inception of this three-pronged model was conducted on only single genes or small clusters of genes, and results obtained from more recent research utilizing the whole genomic sequence suggest at a more complicated structure with six or more clades (Fig. 1) (Kolb et al., 2013).

Notably, there is considerable interstrain variance within the genome of HSV-1. Reference strains differ from each other both *in vitro* and *in vivo*, and also display intrastrain variability (Jones et al., 2019). They differ from clinical strains with regards to, for example, their spread in different tissues and their sensitivity toward acyclovir (Bowen et al., 2019). This genomic variation between circulating strains can be used to trace the spread of the virus from person to person (Pandey et al., 2017), but also to explain the properties of a certain strain (Szpara et al., 2010).

1.1.4 Structure and genome

The HSV virion is comprised of four distinct parts: the core that houses the viral DNA, the capsid that protects the core, a mostly unstructured protein layer known as the tegument, and the envelope, a lipid bilayer the virus is thought to obtain upon exiting the cell (Roizman et al., 2013) (Fig. 2). The HSV genome is made up of linear and double-stranded DNA and is approximately 152,000 base pairs (bp) long for HSV-1 (McGeoch et al., 1988) and approximately 155,000 bp long for HSV-2 (Dolan et al., 1998). Though the genomes of the two HSV types are 83% similar (Dolan et al., 1998), there are marked differences in the locations of endonuclease cleavage sites and the sizes of the proteins they encode that serve to separate the two viruses (Roizman et al., 2013).

The genome of HSV-1 consists of two covalently linked unique components, termed “long” or U_L, which contains 58 genes, and “short” or U_S, which contains 13 genes; furthermore, both components are flanked by repeats on both sides that also contain certain genes (Fig. 3) (Roizman et al., 2013).

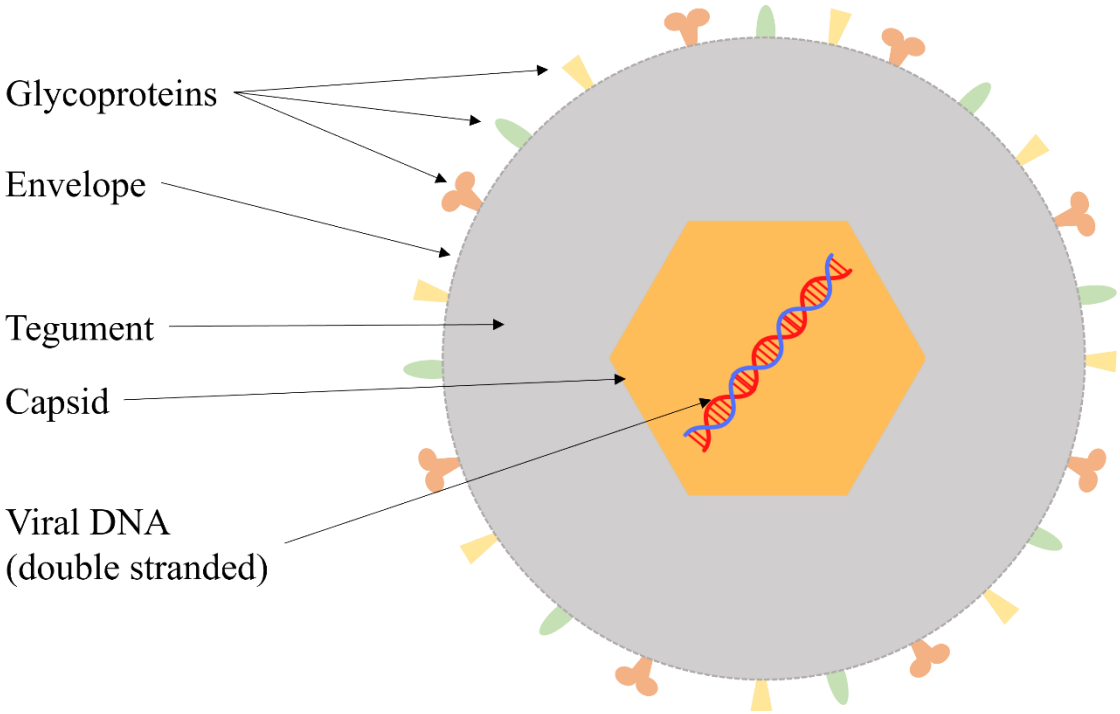

Figure 2. The herpesvirion and its various components. It is comprised of four distinct parts: the DNA-housing core, the protective capsid, a mostly unstructured protein layer known as the tegument, and the envelope, which contains various glycoproteins.

These unique sequences are synthetized in both orientations during replication, which results in four distinct isomers. These include "prototype", or, P in which both U_L and U_S are in their standard configuration, two variations of "inverted", I_S and I_L, in which either the short or long component is inverted, respectively, and I_{S+L}, in which both components are inverted (Mahiet et al., 2012). Currently, HSV's genes have been proven to encode 90 unique transcriptional units, 84 of which code for proteins (Roizman et al., 2013). These protein-encoding genes can be divided into α, β, and γ genes, also referred to as immediate-early, early, and late genes, which code for proteins that regulate viral replication, synthesize and package DNA, and form the virion proteins, respectively (Roizman & Whitley, 2001). This organization, then, helps to structure the cascade-like expression of genes during an infection.

Arguably, one of the most important HSV genes in the context of gene therapy is $\gamma_1$34.5, which is a non-essential gene that encodes infected cell protein 34.5 (ICP34.5) (Roizman et al., 2013). This protein is fundamental for viral neurovirulence, HSV's ability to infect neuronal cells (Chou et al., 1990). Without ICP34.5, the virus becomes attenuated, rendering it unable to cause a lytic infection in the nervous system and decreasing the likelihood of a latent infection (Chou et al., 1990). HSV-1 is very adept at dodging the immune responses of a host body (Melchjorsen et al., 2009), and it has been proven that ICP34.5 has an important role in blocking both adaptive and innate immunity. ICP34.5

can block major histocompatibility complex II responses (Trgovcich et al., 2002), and it has the ability to inhibit protein kinase R function, which not only allows the virus to proliferate but also interferes with type I interferon responses (He et al., 1996, 1997).

Figure 3. The genome of HSV-1, modified from Frampton Jr et al. (2005). It consists of two components, "long", or U_L, and "short", or U_S. Both components are flanked by repeats a and a'. Notably, the repeat pairs, b and b' for U_L and c' and c for U_S, are considered identical. Certain genes of interest have been highlighted in the image. U_L23 codes for thymidine kinase which is essential for the mechanisms of many drugs that seek to treat herpes, U_L27 codes for glycoprotein B which is necessary for the virus's entry into a cell, and U_L29 codes for infected cell protein 8, which is essential in viral replication. U_S7, then, codes for glycoprotein I, which is crucial in untransformed cells. Finally, $\gamma_1$34.5 codes for ICP34.5, which is fundamental for the virus's ability to destroy neuronal cells.

1.1.5 Infection and latency

Transmission by HSV requires contact with mucosal surfaces or broken skin and typically occurs during intimate contact (Roizman et al., 2013). The infection usually spreads through direct contact with a lesion or exchange of infectious bodily fluids, with even asymptomatic individuals able to pass on the virus (Corey & Spear, 1986). An HSV – infection (Fig. 4) will begin with entry into the cell, which is facilitated by heparin sulphate proteoglycans (WuDunn & Spear, 1989) and various cell surface receptors, such as the viral glycoproteins (g) gB, gD, gH, and gL, that allow the virus to merge with the cell membrane (Spear et al., 2000). The capsid will then be transported to the cell's nucleus by dynein along the microtubule network (Kristensson et al., 1986), where the capsid will release the viral DNA (Ojala et al., 2000).

Next, the process of viral transcription will begin. This process can be described as a highly regulated, cascade-like sequence, the first step of which is the derepression and activation of α genes, which will regulate viral replication (Roizman & Whitley, 2013). In the next step, β and γ_1 genes will be expressed, which will result in the synthesis of proteins necessary for the viral DNA replication, and in the third and final step of this process, γ_2 genes will be activated, after which the formation of the capsids and replication of viral DNA will be completed (Amen & Griffiths, 2011). The capsid will be temporarily enveloped when it exits through the nuclear membrane and the virion will finally be

completed as it acquires its envelope after egress (Mettenleiter et al., 2013; Roizman et al., 2013). Though the virus will attempt to delay apoptosis through various means, this process of replication will ultimately lead to the death of the host cell (Yu & He, 2016).

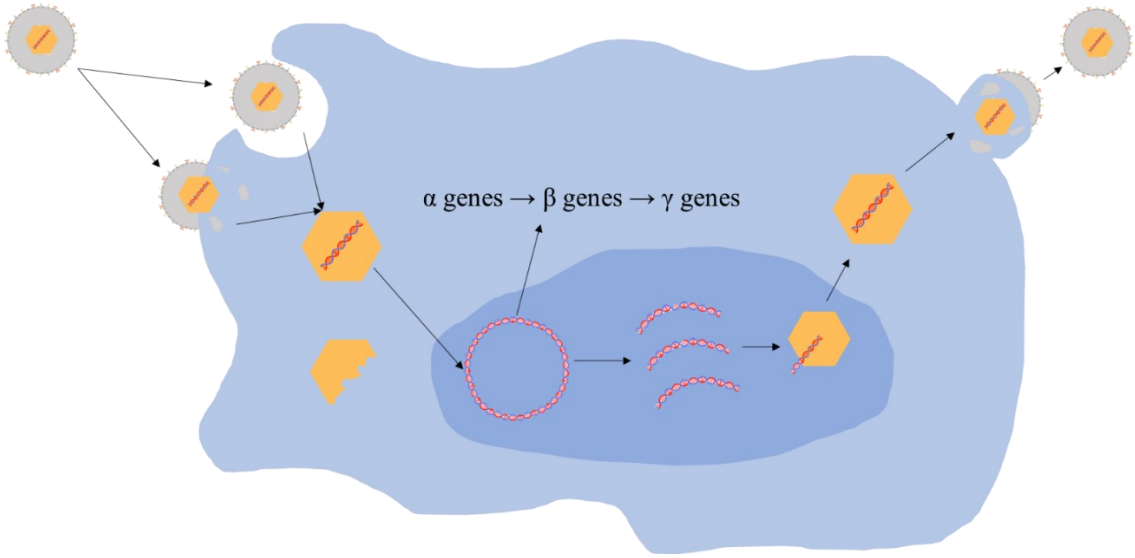

Figure 4. The course of an HSV infection. The infection will begin with entry into the cell, which is facilitated by a merging of the virus particle and the cell membrane. The capsid will be transported to the cell's nucleus where the viral DNA will be released. This is followed by viral transcription, where α, β, and γ genes will be activated in a controlled sequence, which will replicate the viral DNA and produce necessary proteins for the creation of new viral particles. Upon egress, the newly created capsids will be enveloped by the cellular membrane.

Typically, viruses spread by being released, or "shed", from the infected cells, thus spreading to nearby cells, whereupon the process repeats. However, some viruses, such as HSV-1, have also developed the ability to spread laterally from cell to cell, which allows the virions to spread protected from neutralizing antibodies and other defenses (Johnson & Huber, 2002). At the moment, the exact mechanisms of this lateral spread are largely unclear, though it is known that the gEgI complex is essential in the process (Dingwell et al., 1994). Furthermore, the four glycoproteins, B, H/L, and D are known to form a fusion complex that allows viruses to enter the other cell, though this by itself is not enough to facilitate the fusion of cells (Eisenberg et al., 2012). The protein tyrosine phosphatase is known to act as one of the host factors for the rest of the process, but the others are not yet known (Carmichael et al., 2018). Nevertheless, lateral spread has been recognized to be essential in latency, as a virus unable to spread cell-to-cell cannot establish a latent infection (Wang et al., 2010).

Like other herpesviruses, HSV, too, can enter this latent, non-replicative state after the initial lytic infection (Preston & Efstathiou, 2007). This occurs when the virus infects sensory neurons and then travels to their nucleus, where it will often cease to replicate

and persist in an episomal form, which allows the virus to dodge immune responses of the host body (Roizman et al., 2013). The primary site for the latent infection of HSV-1 in humans is typically the trigeminal ganglion (Carton & Kilbourne, 1952; Cushing, 1905), where the latent infection can last for a lifetime. A small fraction of these latent viruses can periodically reactivate, whereby virions will exit the neurons and cause a new lytic infection (Roizman & Whitley, 2001).

1.1.6 Pathology

A typical oropharyngeal HSV –infection is either asymptomatic or causes visible herpetic lesions in the mouth or lips of the patient, the appearance of which can be accompanied by various other symptoms, such as a sore throat and pyrexia (Roizman et al., 2013). As the virus can also enter a latent state and later reactivate, recurring infections are also possible. This reactivation can be caused by many external and internal factors, such as mechanical or psychological stress (Padgett et al., 1998; Sawtell & Thompson, 1992). The number and size of the lesions that appear during the initial infection will indicate its severity, as well as the likelihood of a recurrent infection (Roizman et al., 2013).

The symptoms of genital infection by either HSV-1 or HSV-2 are largely the same, usually consisting of papules and ulcers in the affected area and accompanied by similar systematic symptoms as an oropharyngeal infection (Gupta et al., 2007). Notably, individuals with genital infections caused by HSV-1 are much less likely to have a recurrent infection than those infected with HSV-2 (Lafferty et al., 1987). Nevertheless, these recurrent infections will typically be much milder than the initial infection (Gupta et al., 2007).

In most cases, infections caused by HSV are benign. However, there are notable exceptions to this rule. Neonatal infections, for example, are oftentimes dangerous and can prove fatal, even with drug treatment (Pinninti & Kimberlin, 2018). Neonatal infections are increasingly being caused by HSV-1, and the infection is usually acquired from the mother during childbirth (James & Kimberlin, 2015). The most common form of the neonatal disease is skin, eye, and/or mouth disease, which account for 45% of cases, whereas the other forms of the disease, central nervous system disease and disseminated disease, are less frequent; there is, however, overlap between the different forms and approximately 50% of cases involve the central nervous system in some way (James & Kimberlin, 2015).

Currently, the most important cause of corneal blindness in the world is infectious keratitis, the most common viral cause of which is HSV (Ting et al., 2021). An infection of the eye can cause conjunctivitis, inflammation of the eye, both unilaterally and bilaterally as well as many other symptoms, such as photophobia, oedema of the eyelid, and dendritic lesions (Roizman et al., 2013). Recurrent infections are common and can lead to progressive damage of the eye (Roizman et al., 2013).

Perhaps the most dangerous complication, however, is herpes simplex virus encephalitis. In general, HSV-1 is the most common cause of sporadic encephalitis in the world (Venkatesan, 2015) and has an incidence of 2-4 cases/1,000,000 in Nordic countries (Hjalmarsson et al., 2007). Initial symptoms include an altered state of mind, fever, and seizures, and eventually, the disease can lead to either reduced neurological capacity or death (Bradshaw & Venkatesan, 2016). Indeed, in untreated patients the disease has a 70% mortality rate and only 2.5% of patients are able to regain normal neurological function afterwards (Whitley, 1990). However, encephalitis caused by HSV can be treated with drugs such as acyclovir, which increases the survival rate drastically (Whitley & Gnann, 2002).

It is also important to consider the effect an HSV infection has on immunocompromised individuals, especially as both HSV and human immunodeficiency virus (HIV) are quite common in areas such as sub-Saharan Africa (Dwyer-Lindgren et al., 2019; Looker et al., 2015). Notably, a genital HSV infection, typically caused by HSV-2, can increase the likelihood of contracting HIV significantly (Looker et al., 2020), which explains the interest in developing an HSV-2 vaccine. Furthermore, HSV-infections can also occur during organ transplantation (Lee & Zuckerman, 2019). Infections caused by HSV in immunocompromised hosts are much more severe than in patients with functioning immune systems; for example, the mortality associated with herpes simplex virus encephalitis has been found to be five times higher for immunocompromised individuals (Tan et al., 2012). The virus is also more likely to gain resistance to antiviral therapies (Bacon et al., 2003), and recurrent infections are much more frequent in these patients (Roizman et al., 2013).

1.1.7 Treatment

At the moment, no curative treatment or vaccine for HSV exists, insofar that the latent infection caused by the virus cannot be wholly eliminated (Kim & Lee, 2020), and rather, the disease is controlled with drugs that stop the virus from replicating.

Nucleoside analogs, which include acyclovir and its derivatives, are the first-line treatment for HSV infections as they are both highly specific and have very little toxicity (Roizman & Whitley, 2001). Initially, nucleoside analogs are inert, but upon entering an infected cell, they are converted into monophosphate derivatives by the herpes viral thymidine kinase (TK) and then into triphosphate metabolites by the host cell kinases (Han et al., 2018). After this, the active metabolites begin to inhibit the viral DNA polymerase, which disables viral DNA replication and thus prevents the formation of new virions (Gnann et al., 1983). Furthermore, some nucleoside analogs cause DNA-chain termination by incorporating themselves into the viral DNA (Han et al., 2018). Notably, all nucleoside analogs require functioning copies of the genes U_L23, which encodes the TK, or U_L30, which encodes the catalytic subunit of viral DNA polymerase (Labrunie et al., 2019; Roizman et al., 2013).

Additionally, an exclusively intravenously administered drug, foscarnet, can be utilized to treat severe herpes infections in case of acyclovir resistance (Labrunie et al., 2019). It is a DNA polymerase inhibitor that works by inhibiting the attachment of nucleotide precursors to DNA, which prevents the virus from replicating (Zeichner, 1998). Unlike nucleoside analogs like acyclovir, foscarnet requires no activation and can be used on viruses that lack a normally functioning TK that is necessary for acyclovir; however, it can also cause severe adverse reactions, such as renal failure (Zeichner, 1998).

As a genital HSV-2 infection can increase the likelihood of an HIV infection due to the ulceration it causes (Freeman et al., 2006), it is no surprise that the development of an HSV vaccine is seen as a very important factor for public health, especially in lower-income countries. Currently, most vaccine development projects are focused on preventing or treating HSV-2, but as the two viruses are very similar, HSV-2 vaccines could also prove useful in preventing HSV-1 –infections (Johnston et al., 2016). Vaccine development has proven challenging, however, as animal models do not accurately represent an HSV infection in a human (Kollias et al., 2015) and, especially in the case of prophylactic vaccines, follow-up is a lengthy and costly process (Johnston et al., 2011). Regardless, many prospective vaccines have entered clinical trials. Recent results from

pre-clinical testing of a new HSV-1 –based live attenuated virus vaccine have been promising (Bernstein et al., 2020). Furthermore, recent advances in mRNA vaccines have also seen the development of a trivalent vaccine against genital herpes, currently undergoing preclinical trials (Hook et al., 2022).

1.1.8 RNA interference and HSV

RNA interference is a natural defense mechanism utilized by fungi and invertebrates (Tan & Yin, 2004) and essentially, it describes a process whereby small complementary non-coding RNA, such as siRNA, is used to silence messenger RNA and, by extension, disable the protein synthesis of the threatening pathogen (Levanova & Poranen, 2018). Under normal circumstances, siRNA possess a very limited target sequence, which greatly curtails their effectiveness against viruses with mutated genomes; however, by utilizing so-called "pools" or "swarms" of enzymatically synthesized Dicer substrate siRNAs which cover a target sequence of up to 3000 bp (Nygårdas et al., 2009), this problem can be circumvented. Enzymatically synthetized siRNA swarms represent an emerging, novel form of treatment for HSV infections. These swarms have been proven effective in treating HSV-1 infections *in vitro* (Levanova et al., 2020; Romanovskaya et al., 2012) and *in vivo* (Paavilainen et al., 2017). Crucially, the siRNA-swarms have also been proven efficient against acyclovir-resistant strains of the virus (Kalke et al., 2020).

1.2 Gene therapy

1.2.1 Overview

Gene therapy is an emerging treatment modality, which enables completely new approaches to curing and treating diseases. The European Medicines Agency's (EMA) Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials (2019) defines gene therapy as a method aiming to express a certain transgene, which will then allow for the "regulation, repair, replacement, addition or deletion of a genetic sequence" – that is, the target organ or, in the case of cancer, tumor, can be affected on a genetic level.

All gene therapy methods are contingent on a vector, a "vessel" that can transport the transgene. In most cases, a virus is utilized to fill this role (Wirth et al., 2013). As viruses are very distinct from each other, the gene transfer approach can be carefully optimized

for each target tissue and disease: for example, HSV-1 displays wide tissue tropism, which is especially strong toward the nervous system (Zerboni et al., 2013), while the prevailing tropism of adeno-associated viruses (AAV) depends on their serotype (Drouin & Agbandje-Mckenna, 2013). AAVs are a very popular choice for a viral vector, as they have been proven to be one of the safer options available and the fact that they exert their effect through an episomal form is beneficial for many gene therapy approaches (Naso et al., 2017). They are not the perfect choice for every situation, however: the size of the inserts that can be used with AAV vectors is limited, as only sequences under 5000 bp can be effectively packaged (Naso et al., 2017), though this problem can be circumvented with the use of overlapping vector strategies (Chamberlain et al., 2016). Moreover, the fact that AAV only contains a single strand of DNA slows down the initial transgene expression, though, again, there are ways of alleviating this problem (McCarty et al., 2001). However, perhaps the most prevalent problem AAV's face is the issue of pre-existing immunity: that is, the patient's body will already possess antibodies against the vector, which will severely limit their efficiency (Falese et al., 2017). While HSV-1 vectors will be discussed later in the chapter, it is notable that this problem is absent with HSV vectors.

On the other hand, retroviruses and lentiviruses are RNA viruses, which enables different approaches to gene therapy. Unlike HSVs and AAVs, they always integrate into the host cell's genome and thus modify the patient's genes (Hindmarsh & Leis, 1999). It is important to note that retroviral and even the safer lentiviral vectors are always slightly oncogenic, which increases the likelihood of problematic and potentially long-lasting side effects (Modlich & Baum, 2009). For example, integrating vectors can cause genotoxicity via insertional mutagenesis, which can cause adverse effects such as dysregulation of gene expression or oncogenesis (Ranzani et al., 2013), though this problem has been taken into account when designing newer viral vectors (David & Doherty, 2017). Retroviral and lentiviral vectors are, in the vast majority of cases, used *ex vivo*; that is, the vectors are inserted into their cells in an *in vitro* environment, outside of the patient's body (EMA, 2022b).

As a principle, gene therapy can accommodate a multitude of approaches. One of the most successful methods thus far to have received approval from regulatory agencies is chimeric antigen receptor T (CAR T) therapy, which works by integrating a desired gene into cells grafted from the patients which will then be reintroduced (Ahmad, 2020). In this method, a CAR fusion protein is transduced into T cells collected from patients

(Sermer & Brentjens, 2019). As these modified cells are then reintroduced into the patient, the CAR T cells will bind to specified cancer antigens and activate, which will result in the eradication of antigen-expressing cells (Mohanty et al., 2019). One example of this approach is Abecma, a recently approved lentivirus-based CAR T therapy that is used to treat multiple myeloma (Anderson, 2022). CAR T therapy has also faced adversity, as its administration can often result in a cytokine shock (Brentjens et al., 2013) and can also carry a significant risk of neurotoxicity and CAR T cell-related encephalopathy syndrome (Sermer & Brentjens, 2019). However, increased understanding of the therapy's adverse effects has made the treatment of these reactions easier and more efficient (Brudno & Kochenderfer, 2019).

Furthermore, *ex vivo* –mediated retroviral therapy or non-integrating vectors, such as AAV or HSV, can be used to repair genes to treat, for example, hereditary diseases that are caused by genetic mutations. Oftentimes these mutations can cause a deficiency or even total lack of a critical protein, and by inserting a functioning version of this gene into the patient, either *ex vivo* or directly depending on the vector, the production of these necessary proteins can be induced (Booth et al., 2016). For example, Strimvelis is a retrovirus-based gene therapy product that is used to treat severe combined immunodeficiency caused by adenosine deaminase (ADA) deficiency. The disease is a hereditary, often fatal metabolic disorder that causes immunodeficiency due to mutations in the ADA-gene, which causes a deficiency in the enzyme (Ferrua & Aiuti, 2017). Strimvelis works by introducing a functioning copy of the ADA-gene into grafted CD34+ cells via a retroviral vector (Booth et al., 2016). The modified cells are then introduced back into the patient's bone marrow and will eventually begin to generate lymphocytes that can produce ADA normally, enhancing the patient's immune response (Ferrua & Aiuti, 2017).

Today, cancers are the most important indication for gene therapy, by a large margin (Wirth et al., 2013). In addition to the aforementioned CAR T therapy, one approach to treating cancer with gene therapy is to attack the tumor directly with specifically modified viruses, a method known as oncolytic virotherapy. This type of therapy was first attempted in the first half of the 20th century, but researchers soon discovered that while the viruses were initially efficient in reducing the tumors, this effect was only temporary (Newman & Southam, 1954). However, the myriad advancements made in the field since then have made oncolytic therapy a viable avenue of research once again. Typically, this approach is utilized by injecting replication-competent viruses, such as oncolytic HSV or

adenoviruses, straight into the tumor (Mullen & Tanabe, 2002). The viruses will then begin to replicate, which will eventually lead to the lysis of their host tumor cells (Mullen & Tanabe, 2002). This is especially effective, as it has been proven that the body's defense mechanisms against viral infections, such as interferon-β signaling, are notably impaired in cancerous cells (Lurie & Platanias, 2005). Oncolytic viruses can also induce the activation of the host body's immune system, as well as confer an improved response to chemotherapy (Mullen & Tanabe, 2002). An important facet of oncolytic viruses is that they can be rendered specific to the tumor cells by either adding tumor-specific promoters to critical loci in their genome or by removing the genes that allow the viruses to replicate in non-neoplastic cells (Mullen & Tanabe, 2002), such as has been done with the oncolytic HSV-1 -based talimogene laherparepvec (T-vec) (Johnson et al., 2015). As such, theoretically, the virus cannot replicate in the host's normal cells and will only kill tumor cells. Thus far, T-vec is the only oncolytic virus to have been approved in the western world (Table 2).

1.2.2 Current landscape

Though the technology is still in its relative infancy, multiple different kinds of therapies have already received approval both in Europe and the USA (Table 2). Especially in Europe, many of these therapies have received an orphan status, meaning that they are designated for exceedingly rare conditions (EMA, 2022b). Around half of the available therapies are indicated toward cancer, with CAR T –cells being the most common approach. Various kinds of viruses have been used as viral vectors, with AAVs, retroviruses, and lentiviruses being the most ubiquitous (David & Doherty, 2017).

Table 2. Currently available gene therapies in Europe and the United States according to EMA (EMA, 2022a) and the FDA (FDA, 2022).

Product	*Approved (EMA)*	*Approved (FDA)*	*Indication*	*Vector*	*Mechanism*
Abecma	August 2021	March 2021	Multiple myeloma	Lenti-virus	CAR T –therapy
Breyanzi	N/A	February 2021	Large B-cell lymphoma	Lenti-virus	CAR T –therapy
Imlygic	December 2015	October 2015	Melanoma	HSV-1	Oncolytic virus
Kymriah	August 2018	August 2017	Blood cancer	Lenti-virus	CAR T –therapy
Libmeldy	December 2020	N/A	Metachromatic leukodystrophy	Lenti-virus	Ex vivo -delivery of functional gene
Luxturna	November 2018	December 2017	Retinal dystrophy	AAV	Delivery of functional gene
Strimvelis	April 2016	N/A	Adenosine deaminase deficiency	Retro-virus	Ex vivo -delivery of functional gene
Tecartus	December 2020	July 2020	Mantle cell lymphoma	Retro-virus	CAR T –therapy
Yescarta	August 2018	October 2017	B-cell lymphoma	Retro-virus	CAR T –therapy
Zolgensma	May 2020	May 2019	Spinal muscular atrophy	AAV	Delivery of functional gene
Zynteglo	May 2019	N/A	Beta-thalassemia	Lenti-virus	Ex vivo -delivery of functional gene

1.3 HSV-1 as a gene therapy vector

1.3.1 Advantages of HSV-1 as a vector

HSV-1 harbors a wide array of properties that make it an attractive prospect for gene vector development, and for this reason, it has been one of the most widely researched vector candidates (Wirth et al., 2013). HSV-1 has wide tissue tropism (Buthod et al., 1987) and is safe even when replication-competent (Manservigi et al., 2010), which are both very useful properties in terms of vector development. Furthermore, its genome is episomal, which means that it will not integrate to the host's genome unlike retroviral vectors, and as such, any HSV-vector is unlikely to cause undesired mutations in the patient (Roizman, 1996). Unlike, for example, AAV-based vectors, HSV-based vectors can support insertions of up to 30 000 bp, as the HSV genome is particularly large and capable of withstanding large insertions (Ventosa et al., 2017) in addition to containing

many non-essential genes, which can be replaced with large transgenes without severely affecting the functionality of the virus (Roizman, 1996). Furthermore, HSV's genome has been sequenced and, as a result, its neurovirulence gene has been identified (Chou et al., 1990). This enables researchers to modify the gene in a way that will adjust the virus's replication and render the virus safe for use in human gene therapy, for example by restricting its ability to replicate in all non-neoplastic cells (Liu et al., 2003).

As already discussed, HSV-vectors' safety is greatly enhanced by the fact that acyclovir is so efficacious in stopping the spread of the infection, and the drug can be essentially used as an "emergency brake", should something go wrong with the vector. HSV-1 has also shown great potential in the treatment of neurological disorders, with replication defective HSV-1 –vectors already proven to be effective in treating neurodegenerative diseases and chronic pain in man (Fink et al., 2011), and the field has already seen further developments with the emergence of regulatable vectors (Wu et al., 2011) and HSV-1 amplicons (Spaete & Frenkel, 1982). One additional important advantage HSV-based vectors boast is the possibility of repeated dosing. Unlike other vector types, such as AAVs, HSV-1 –vectors can be dosed repeatedly without the patient forming any resistance to the vector (Chahlavi et al., 1999).

Notably, one of the few virus-mediated genetic therapies available, and the first approved oncolytic therapy in the Western world, is an HSV-1 –vector (Johnson et al., 2015). This melanoma treatment, T-vec, is marketed as Imlygic. As an oncolytic virus, it has been modified to be tumor selective, meaning that it does not harm cells of the nervous system, thus removing the risk of encephalitis, and rather only selectively replicates in cancerous cells (Bartlett et al., 2013). As such, it acts as a precision weapon against a tumor with minimal damage to the surrounding tissue. Moreover, it also possesses a transgene for granulocyte-macrophage colony-stimulating factor, which enhances the vector's ability to activate the body's own immune system against the tumor (Johnson et al., 2015; Liu et al., 2003). The therapy is repeatedly administered directly into the tumor and has been proven both effective and safe for the patients (Kaufman et al., 2014). T-vec viruses are replication competent and manufactured in Vero cells (EMA, 2015), thus making them relatively affordable: in the USA, treatment with Imlygic costs around $65,000 (Amgen, 2015) and in Finland only 1450-11600€ per treatment (Pharmaca Fennica, 2022). In stark contrast, the prices of other gene therapy treatments range from $373,000 per treatment for Yescarta (Drugs.com, 2021) to $2,100,000 per treatment for Zolgensma (Drugs.com, 2020).

1.3.3 Developmental pipeline

According to a search conducted in the ClinicalTrials.gov database (NIH, 2022), as of 8.3.2022, there are 17 different HSV-1 -based vectors in clinical phases, a vast majority of these vectors being oncolytic viruses (Fig. 5). The therapy currently furthest along in the developmental pipeline is Beremagene geperpavec or B-vec (Table 3). It is a topical gene therapy for the treatment of the skin disease recessive dystrophic epidermolysis bullosa and it has currently completed Phase III testing following promising results from phase 1/2 trials (Krystal Biotech, 2021). Similarly, both G207 (Friedman et al., 2021) and HF-10 (Hirooka et al., 2018) have exhibited sufficiently favorable safety profiles to proceed to Phase II. Perhaps the most unique one of the prospective vectors is NP2, however. NP2 expresses the human proenkephalin gene, the protein products of which have been proven to inhibit pain signaling (Dickenson & Kieffer, 2006). Phase I testing suggested that NP2 is not only safe but also works as a neuronal analgesic when used in sufficiently high doses (Fink et al., 2011).

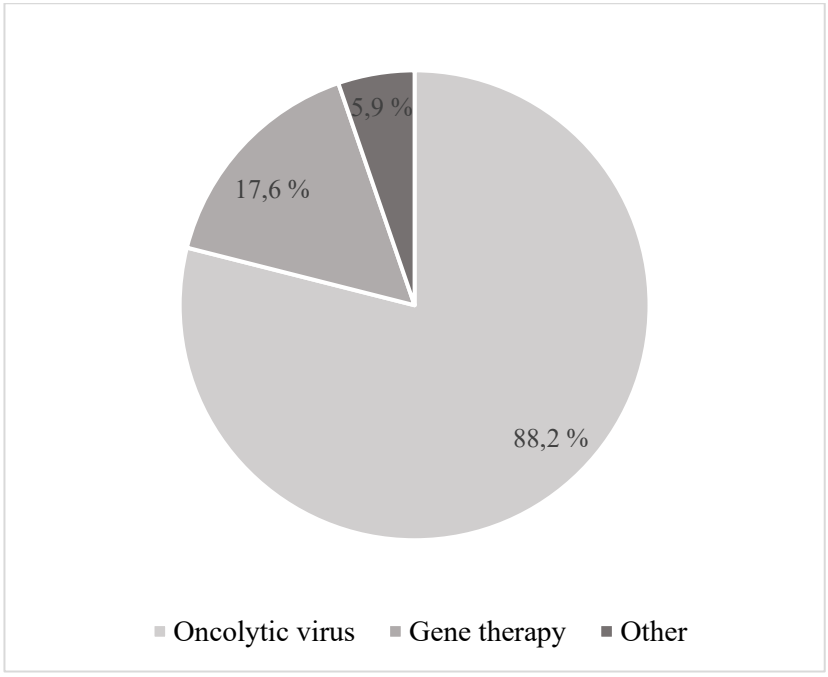

Figure 5. HSV-based gene therapies in clinical development by indication.

Table 3. HSV-1 -based vectors in clinical development.

Virus	Function	Status	Study initialized	Identifier
B-vec	Gene repair	Phase III	2021	NCT04917874
C134	Oncolytic virus	Phase I	2018	NCT03657576
C5252	Oncolytic virus	Phase I	2021	NCT05095441
G207	Oncolytic virus	Phase II	2020	NCT04482933
G47Δ	Oncolytic virus	Phase II	2014	UMIN000015995
HF-10	Oncolytic virus	Phase III	2017	NCT03153085
HSV1716	Oncolytic virus	Phase I/II	2012	NCT01721018
KB105	Gene repair	Phase I/II	2019	NCT04047732
KB407	Gene repair	Phase I	2021	NCT05095246
M032	Oncolytic virus	Phase I/II	2021	NCT05084430
NP2	Other	Phase II	2011	NCT01291901
NV1020	Oncolytic virus	Phase I/II	2018	NCT00149396
ONCR-177	Oncolytic virus	Phase I	2020	NCT04348916
OrienX010	Oncolytic virus	Phase I	2019	NCT04206358
RP1	Oncolytic virus	Phase I	2020	NCT04349436
RP2	Oncolytic virus	Phase I	2020	NCT04336241
RP3	Oncolytic virus	Phase I	2021	NCT04735978
rQNestin	Oncolytic virus	Phase I	2017	NCT03152318
VG161	Oncolytic virus	Phase I	2021	NCT05162118

2. Results

2.1 Determination of replication characteristics

2.1.1 Determination of cell bound and shed virus concentrations

In order to determine the production rate of both released (shed) virus and cell bound virus, the clinical strains were used to infect four different cell lines, and at 24 hours post-infection (hpi), a plaque assay was utilized to quantify the virus titers. The titer was presented as plaque-forming units (PFU), that is, particles capable of forming plaques, per ml. Cell bound titer was determined from lysed cells, while released virus titer was determined from the supernatant (Fig. 6). Moreover, a released-to-cell bound ratio was calculated for each strain in each cell line (Fig. 7). Finally, the results from each strain were compared with those obtained with the reference strain 17+.

In Vero African green monkey kidney cells (Fig. 6 A) the shed titers for the clinical strains ranged from $4.96*10^3$ PFU/ml to $6.16*10^6$ PFU/ml, with an average of $1.37*10^6$ PFU/ml and a SD of $1.46*10^6$ PFU/ml. The cell bound titers ranged from $1.48*10^6$ PFU/ml to $6.21*10^7$ PFU/ml with an average of $1.67*10^7$ PFU/ml and SD of $1.27*10^7$. Of the clinical strains, V22 possessed the highest released titer and V14 the highest cell bound titer while V19 had both the lowest shed titer and cell bound titer. Clinical strains V2, V9, V10, V12, V13, V17, V18, V19, V20, V21, V30, V32, and V33 had significantly lower shed titer than the reference strain 17+, while only strain V22 had a higher titer. Cell bound titers were more level in Vero cells, as only strains V17, V19, V32, V33, and the reference virus H1052 had a significantly lower titer than 17+, while V14 possessed a significantly higher titer than 17+. When comparing the ratio of released virus to cell bound virus, no strain possessed a higher proportion than 17+, but strains V2, V9, V12, V13, V19, V20, V21, V26, and V30 had a significantly lower percentage of shed virus (Fig. 7 A).

In WM1799 human melanoma cells (Fig. 6 B) the shed titers for clinical strains ranged from $1.38*10^4$ PFU/ml to $8.36*10^5$ PFU/ml, with an average of $1.90*10^5$ PFU/ml and SD of $1.86*10^5$ PFU/ml. The cell bound titers ranged from $4.37*10^5$ PFU/ml to $2.35*10^7$ PFU/ml with an average of $4.84*10^6$ PFU/ml and SD of $4.85*10^6$ PFU/ml. V12 and V1 had the highest released and cell bound titers, respectively, while V30 had both the lowest shed and cell bound titers. Only the clinical strains V2, V30, V32 and V33 and the

reference strain KOS differed significantly from 17+, all of them having a lower shed virus titer. On the other hand, no strain possessed a significantly different cell bound titer from 17+. This was also true of the released-to-cell bound ratio, as no strain differed significantly from 17+ (Fig 7 B).

In U373MG astrocytoma cells (Fig. 6 C) the shed titers for clinical strains ranged from $3.50*10^2$ PFU/ml to $1.98*10^5$ PFU/ml, with an average of $2.82*10^4$ PFU/ml and SD of $4.31*10^4$ PFU/ml. The cell bound titers ranged from $1.63*10^5$ PFU/ml to $5.00*10^6$ PFU/ml with an average of $1.63*10^6$ PFU/ml and SD of $1.17*10^6$ PFU/ml. V6 had the highest shed titer while V18 had the lowest, whereas V5 had the highest cell bound titer and V19 the lowest. No strains had a significantly lower released titer than 17+, however strains V3, V4, V5, V6, V7, V11, and V12 had a significantly higher titer. The situation was similar with cell bound titers, as only H1052 possessed a significantly lower titer than 17+. On the other hand, strains V1, V4, V5, V6, V7, V12, V26, V35, and V36 had significantly higher titers. When compared to 17+, only two strains, V7 and V12, possessed a significantly higher percentage of shed virus and no strains had a significantly lower percentage (Fig. 7 C).

Finally, in HCE human corneal epithelial cells (Fig. 6 D), the shed titers for clinical strains ranged from $1.30*10^3$ PFU/ml to $6.44*10^4$ PFU/ml, with an average of $1.63*10^4$ PFU/ml and SD of $1.61*10^4$ PFU/ml. The cell bound titers ranged from $7.20*10^4$ PFU/ml to $1.53*10^6$ PFU/ml with an average of $4.81*10^5$ PFU/ml and SD of $3.65*10^5$ PFU/ml. V4 had the highest released virus titer, while V12 had the highest cell bound titer. V2 had both the lowest released and cell bound titers. Clinical strains V4, V5, V12, V13, and V19 had a significantly higher shed titer than the reference strain, while strains V2, V18, V29 and all three other control strains had a significantly lower shed titer. Clinical strains V4, V5, V7, V12, V24, and V36 had a significantly higher cell bound titer than 17+, while only the control strain H1052 possessed a significantly lower titer than 17+.When comparing the ratio of shed to cell bound virus, no strain proved to possess a higher proportion of shed virus than 17+, though strains V29 and V36 had a significantly lower percentage of shed virus (Fig. 7 D).

Across all cell lines, strains V4, V5, and V12 were the most prolific shedders, displaying a significantly higher titer than 17+ in both U373MG cells and HCE cells. The lowest-shedding strain was V2, which, when compared with 17+, had a significantly lower shed titer in Vero cells, HCE cells, and WM1799 cells.

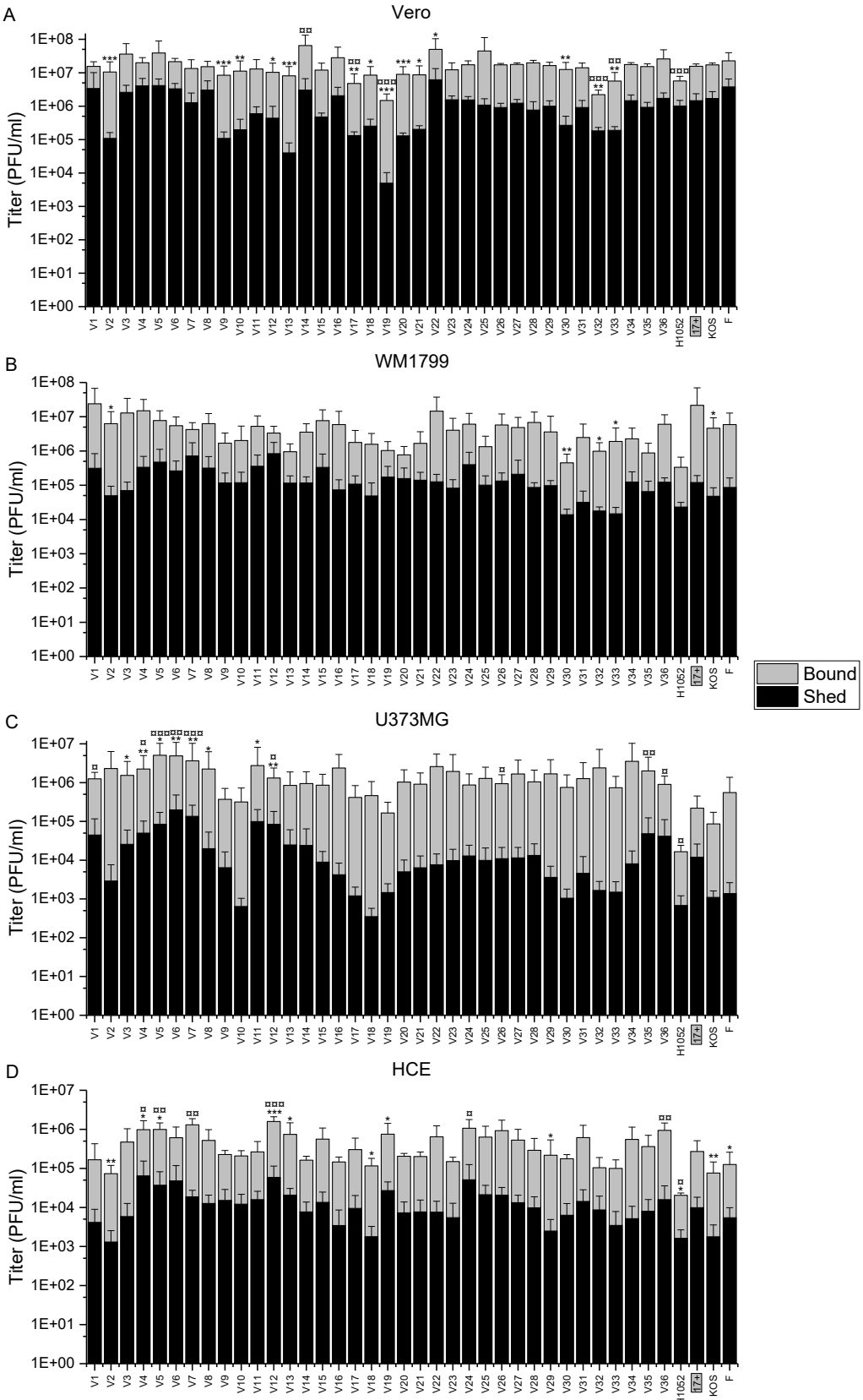

Figure 6. Shed and cell bound titers in different cell lines. African green monkey kidney (Vero, A), human melanoma (WM1799, B), astrocytoma (U373MG, C), and corneal epithelial (HCE, D) cells were infected with each strain in a 96-well format. At 24 hpi, a plaque assay was used to calculate the titers separately from the supernatant to get the released virus titer, and from lysed cells to get the cell bound virus titer. The bars represent the titers in PFU/ml, with the black bars depicting the shed titer and the grey bars the cell bound titer. The whiskers represent the standard

deviation of the mean (N≤8 per treatment, data from two individual experiments). The reference virus used for comparisons, 17+, has been highlighted with a grey background, and was used to calculate p-values (*, p<0.05; **, p<0.01; ***, p<0.001 for released virus, and ¤, p<0.05; ¤¤, p<0.01; ¤¤¤, p<0.001 for cell bound virus).

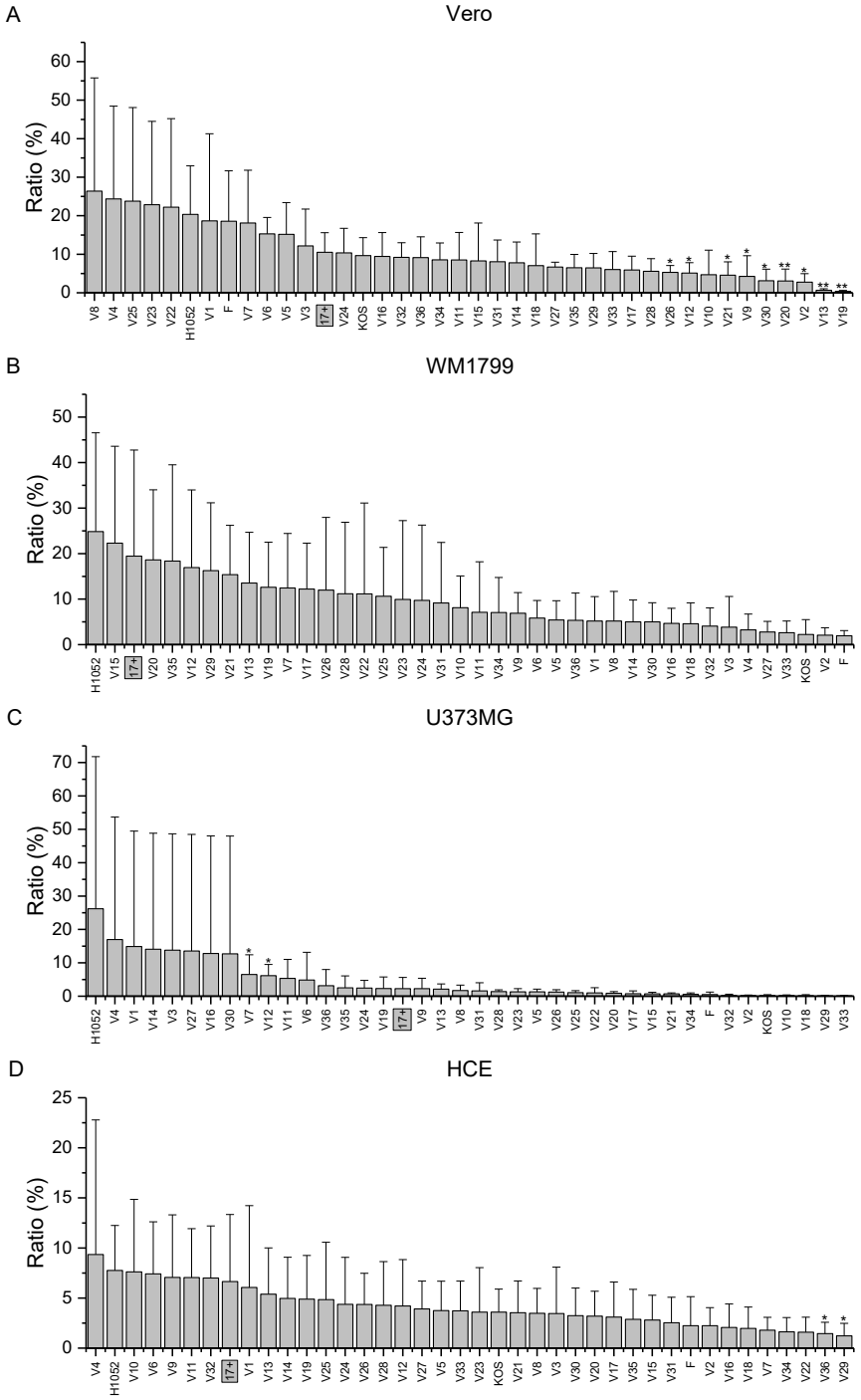

Figure 7. Relative released virus in different cell lines. Shed and cell bound titers were compared with each other, and the relative shed of each strain was determined in Vero (A), WM1799 (B), U373MG (C), and HCE (D) cells. The bars represent the titers in percentage of shed titer compared to the total, combined titer and the whiskers represent the standard deviation of the mean (N≤8 per treatment, data from two individual experiments). The reference virus used for comparisons, 17+, has been highlighted with a grey background. The p-values were calculated against the shed/cell bound ratio of the reference strain 17+ (*, p<0.05; **, p<0.01).

Other low-shedding strains were V18, V30, V32, and V33 in addition to the reference strain KOS, each of which had significantly lower shed titers than 17+ in two cell lines. Strains V4, V5, V7, V12, and V36 had a significantly higher cell bound titer than 17+ in two separate cell lines.

The recombinant virus strain H1052 was the only one to have a significantly lower cell bound titer in more than one cell line, with the difference being significant in Vero cells, U373MG cells, and HCE cells. Strains V2, V9, V12, V13, V19, V20, V21, V26, and V30 had a significantly lower ratio of shed virus to cell bound virus in Vero cells while the strains V29 and V36 had a significantly lower ratio of shed virus in HCE cells. Strains V7 and V12 had a significantly higher proportion of shed virus in U373MG cells. There were no significant differences in terms of shed to cell bound ratios in WM1799 cells. The only clinical strain to have a significantly different shed to cell bound ratio in two cell lines was V12. Its ratio was significantly lower than 17+ in Vero cells but significantly higher than 17+ in U373MG cells.

2.1.2 Growth curve

Two growth curves were compiled during the experiment: one with all the clinical strains, and another one with a curated set of strains considered to be interesting (please refer to chapter 4.7 for inclusion criteria). Vero cells were infected with 5 MOI of each virus in a 96-well format for the larger growth curve, and in a 24-well format for the "strains of interest" –growth curve. In both cases, the initial virus dilution was collected and referred to as the 0 h time point. Subsequent samples were obtained from the supernatant at 6, 24, 48, and 72 hpi, which were then used to determine the titers at each time point.

The used viral dilutions were titered, and V5 had the highest titer at $6.75*10^4$ PFU/ml, while KOS possessed the lowest titer at $9.00*10^3$ PFU/ml, which was notably less than the other viruses (Fig. 8). At the 6 hpi eclipse phase, the titer of all viruses had reached the minimum concentration. At 24 hpi, the titer had decreased for strains V2, V3, V4, V8, V11, V12, V13, V15, V16, V18, V19, V27, V29, V33, V35, and KOS, remained the same for V9, and increased for the rest when compared to the previous time point. V5 had the highest titer at $1.48*10^4$ PFU/ml and KOS the lowest for at 50 PFU/ml. However, at the next time point, 48 hpi, all strains displayed an increase in their titer: V22 now had the highest titer at $5.88*10^4$ PFU/ml, while V2 had the lowest at $7.50*10^2$ PFU/ml. Finally, at 72 hpi, the virus concentration had increased for only 4 clinical strains, V2, V11, V29,

V35, and the reference viruses, and remained unchanged for V30 and V36. At the final time point, F had the highest titer at $5.25*10^4$ PFU/ml. Of the clinical strains, V34 had the highest titer at $3.5*10^4$ PFU/ml and V19 the lowest titer at 500 PFU/ml.

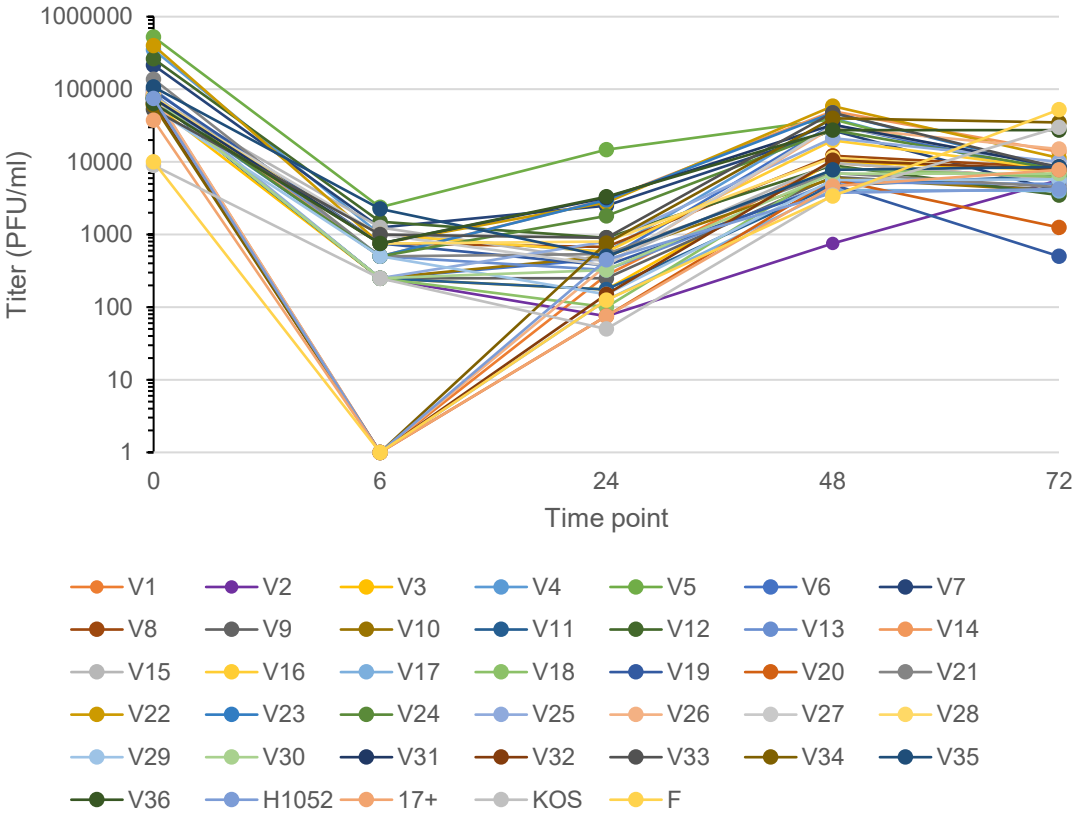

Figure 8. Growth curve for all clinical strains and reference strains. Vero cells were infected with approximately 5 MOI of each virus in a 96-well format. The initial virus dilution created to infect the cells was used as the 0 h time point, while subsequent samples were obtained from the supernatant at 6, 24, 48, and 72 hpi. A plaque assay was then used to determine the titers at each time point. The titers are represented with a logarithmic scale.

The general shape of the growth curve was the same for most of the clinical strains. The titer of each strain had decreased after the washes at the 6 hpi time point, after which it started to increase for around half of the strains. The titer still decreased for strains V2, V3, V4, V8, V11, V12, V13, V15, V16, V18, V19, V27, V29, V33, V35, and the reference strains, while it stayed the same for V9. At 48 hpi, however, the titer had increased across all strains, though none of them reached the initial infection titer at this time point. Finally, at 72 hpi, the titers waned for most of the strains, with the exception of V2, V11, V29, V35, and the reference strains. Though the final titer of the reference strains KOS and F exceeded the initial infection titer, this was not true for any of the clinical strains.

In addition to the reference strains H1052 and 17+, the clinical strains V3, V4, V5, V6, V7, V11, V12, V16, V22, and V27 were included in the second growth curve (Fig. 9). The initial 0 hpi titers were relatively even, with V5 once again possessing the highest initial titer at 4.50 PFU/ml, while V4 had the lowest initial titer at 1.00 PFU/ml. As with the larger growth curve, the titer of each virus had dropped at the 6 hpi time point, with V3 having the highest titer at 0.068 PFU/ml and H1052 the lowest at 0.003 PFU/ml. At the 24 hpi time point each strain's titer had grown, with H1052, in particular, showing a drastic increase. At this time point, V5 had the highest titer at 6.06 PFU/ml and V12 the lowest at 0.321 PFU/ml. The titers had continued to increase at 48 hpi, with the exception of H1052. V6 had the highest titer at 13.05 PFU/ml, while H1052 predictably had the lowest titer at 1.215 PFU/ml. At the final time point, 72 hpi, the titer for all strains began to decrease. V6 still possessed the highest titer at 7.45 PFU/ml and H1052 the lowest at 0.181 PFU/ml.

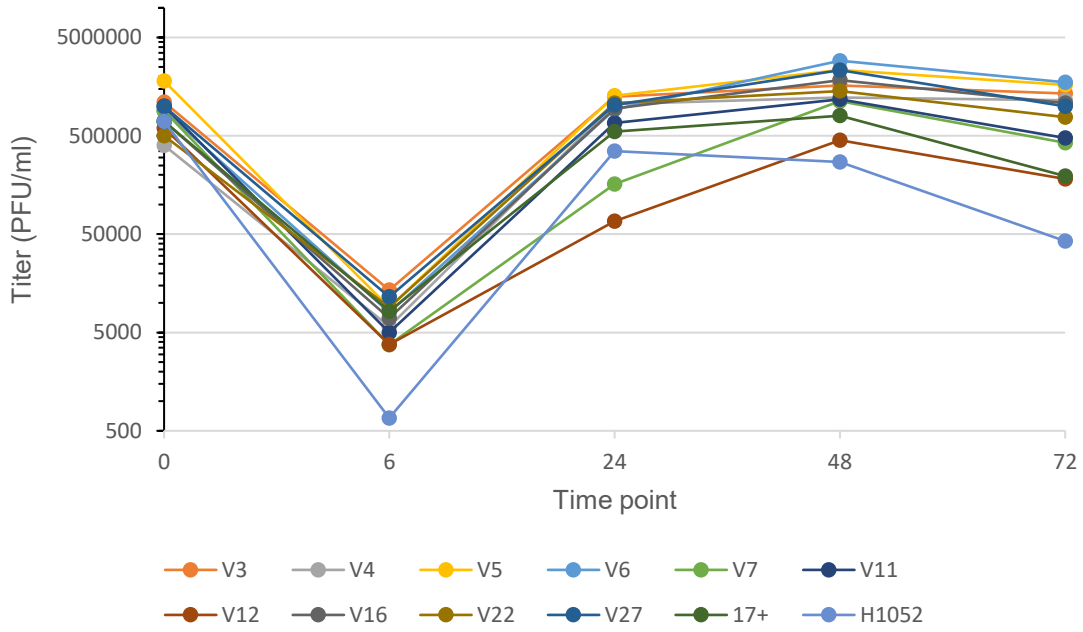

Figure 9. Growth curve for a curated group of strains with unique characteristics. Vero cells were infected with approximately 5 MOI of each virus in a 24-well format. The initial virus dilution was used as the 0 h time point, and supernatant samples were obtained at 6, 24, 48, and 72 hpi. The titers at each time point were determined with a plaque assay. The titers are represented with a logarithmic scale.

Most of the strains used in the experiment displayed relatively similar growth curves, in that their titer decreased after the wash, after which it started to steadily increase. All strains, with the sole exception of H1052 which had its peak at 24 hpi, displayed their highest post-infection titer at 48 hpi, after which the concentration started to decrease.

Unlike in the previous setting (Fig. 8), all of the clinical strains and the reference strain 17+ reached and exceeded the initial infection titer. This occurred at 48 hpi for V7 and V12 and already at 24 hpi for the others. However, the reference strain H1052 never reached the initial infection titer.

2.1.3 Plaque morphology

Vero cells infected with each virus strain were observed during the infection and imaged at 72 hpi (Fig. 10). All 36 clinical strains produced plaques in Vero cells. However, none of the infections produced strictly uniform plaques, and as such, no noticeable differences could be distinguished between the plaque morphologies of any of the strains of interest.

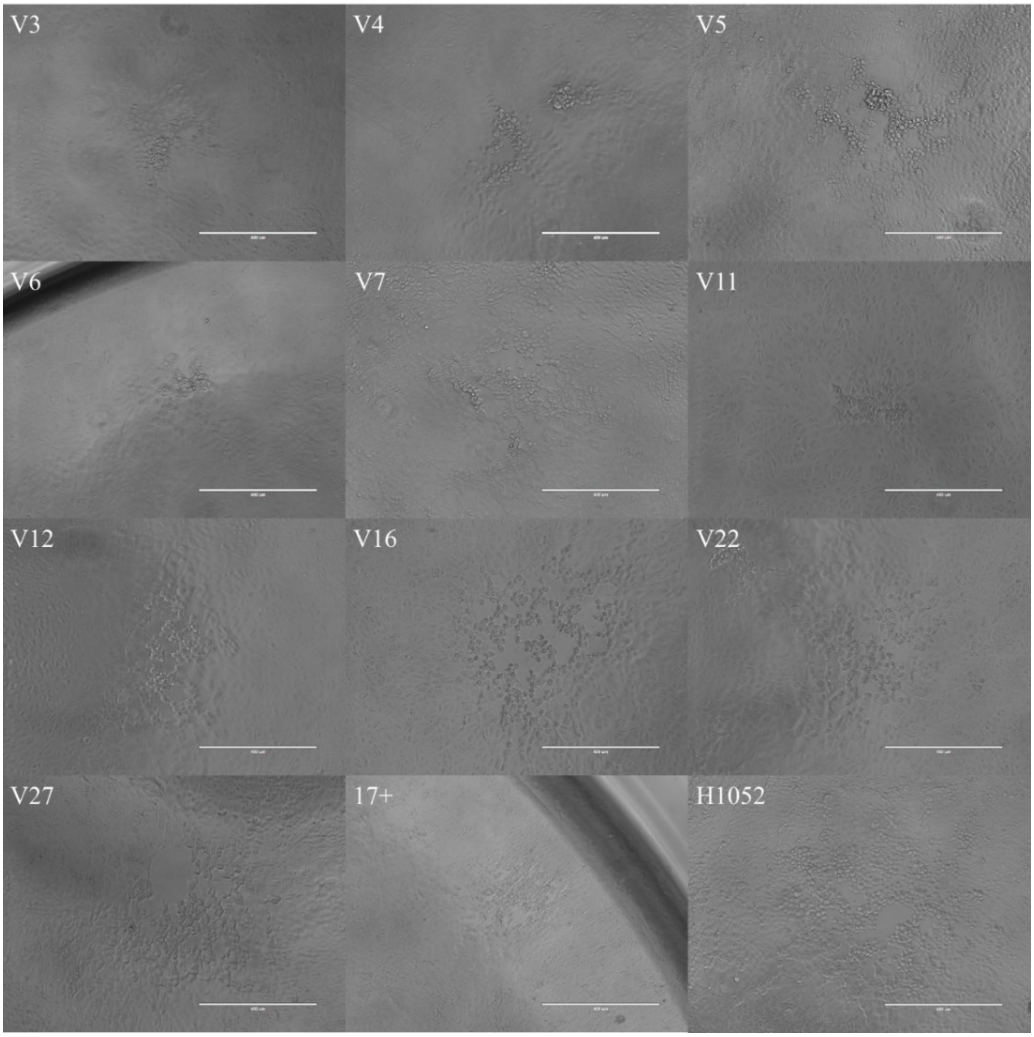

Figure 10. Transmitted light microscopy images of virus plaques from the strains of interest, taken at 72 h post-infection. The infection was conducted in Vero cells. The scale bars depict the magnification of the images.

2.1.4 Determination of oncolytic effect

The oncolytic effect of each strain was studied across three cancerous cell lines by infecting them with approximately 2 MOI of each virus. The oncolytic effect was estimated by comparing the viability of the cells infected with the viruses to an uninfected control at 96 hpi (Fig. 11). All tested strains were oncolytic in all cell lines, though the effect was far more pronounced in SW480 adenocarcinoma cells and Raji lymphoma cells, where cell viability dropped to under 50% for all clinical strains, whereas in U373MG cells viability dropped 10–40% with the used virus dose and timeframe.

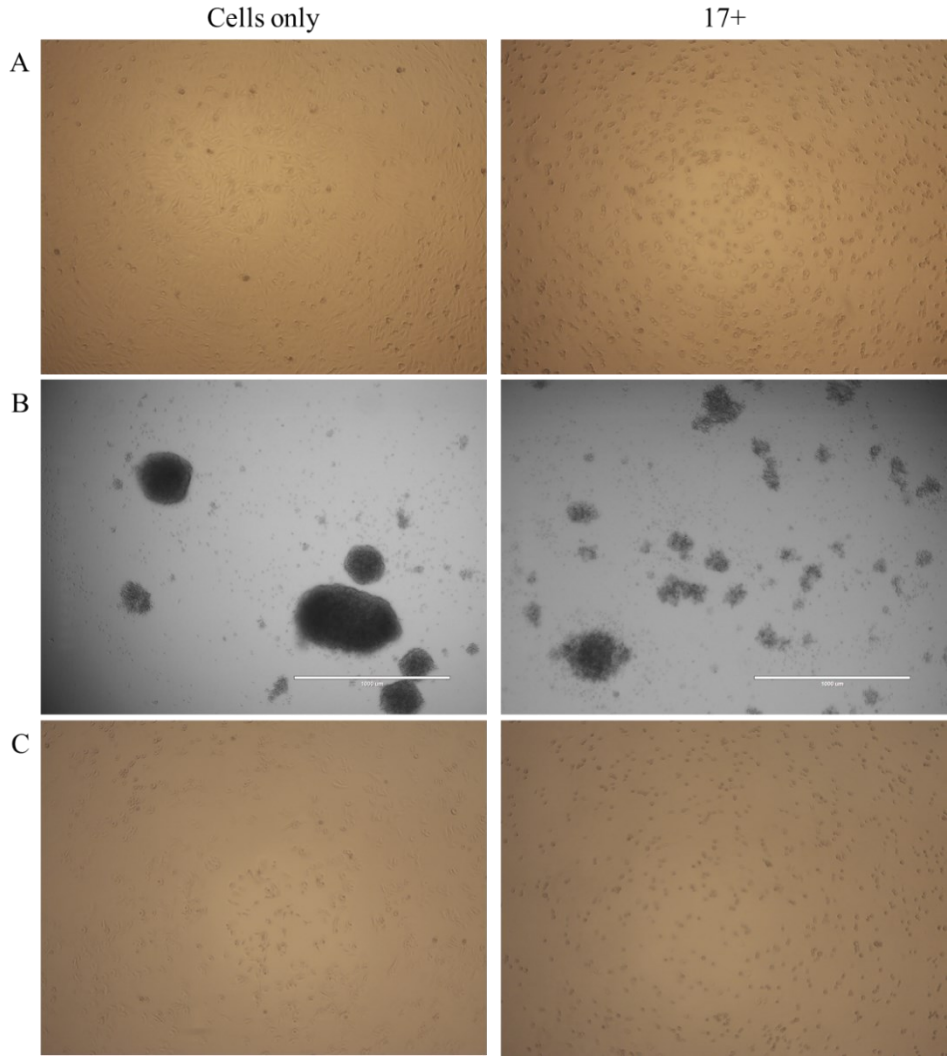

Figure 11. Microscope images of the cells only –control and 17+ -treated cells in U373MG (A), Raji (B), and SW480 (C) cells. The images were taken at 96 h post-infection. The scale bar represents the magnification of the images.

Each strain was compared to the reference virus 17+ within the cell line. In U373MG cells (Fig. 12 A) statistically significant differences were rare. Strains V10, V14, and V17 caused a significantly lower drop in cell viability than 17+, while strains V13, V27, and V35 had a significantly higher decline in viability.

In SW480 cells (Fig. 12 B) the reference strains H1052 and KOS displayed a significantly lower decrease in cell viability compared to 17+. The only strains not to have a significantly higher oncolytic effect than 17+ were V17 and the reference strain F.

In Raji cells (Fig. 12 C) clinical strains V2, V4, V5, V29, V31, and V35, and the reference strain H1052 had a significantly more potent oncolytic effect than 17+, while in addition to the reference strains H1052 and KOS, the clinical strains V9, V10, V11, V13, V18, V19, V21, V23, V25, V26, and V30 caused a significantly smaller drop in viability.

Across all cell lines, V35 showed the most oncolytic potential by rendering the cells significantly less viable than 17+ in all three cell lines. Strains V2, V4, V5, V27, V29, and V31 all showed significantly more oncolytic effect in two cell lines. On the other hand, the reference virus KOS was the least oncolytic, as the cells infected with the virus were significantly more viable than cells infected with 17+ in all SW cells and Raji cells. The least oncolytic clinical strain was V17, which had a significantly higher viability than 17+ in U373MG cells and displayed no other significant differences. Similarly, the only significantly different result for the reference strain F occurred in Raji cells, where the oncolytic effect was found to be significantly lesser than 17+. Lastly, though V10 was significantly more oncolytic in SW480 cells, it was significantly less oncolytic than 17+ in both U373MG and Raji cells.

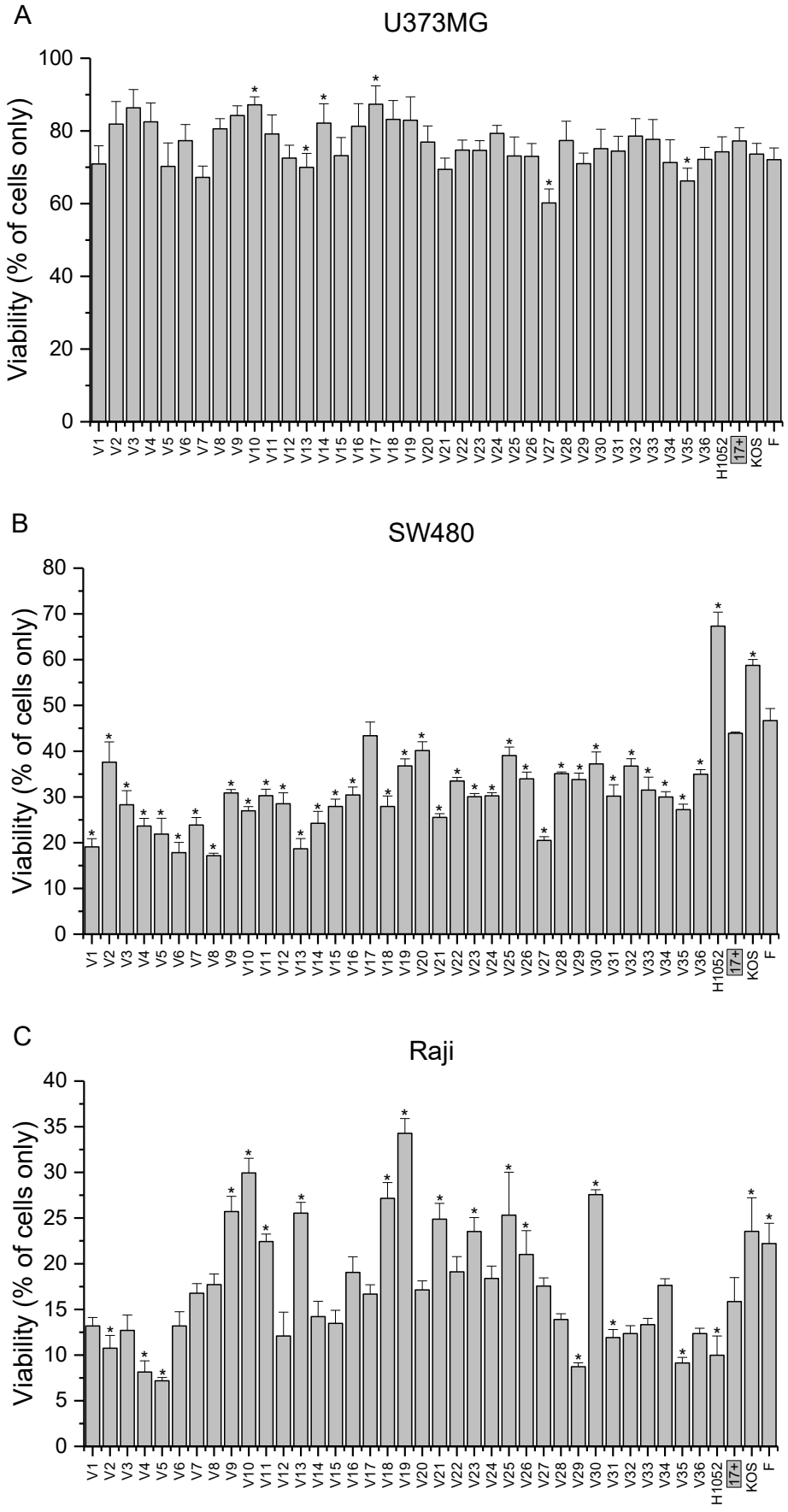

Figure 12. Measured viability of cells infected with the virus strains in comparison to the cells only –control. The oncolytic effect of each strain measured across the three cancerous cell lines

U373MG (astrocytoma, A), SW480 (adenocarcinoma, B), and Raji (lymphoma, C). The cells were infected with approximately 2 MOI of each virus and the oncolytic effect was estimated by comparing the viability of the cells infected with the viruses to an uninfected cells only –control at 96 hpi. The bars represent the percentile viability of the cells treated with each virus in comparison with the uninfected cells only control and the whiskers represent the standard deviation of the mean (N=4 per treatment). The p-value (*, $p<0.05$) of the relative inter-cell line oncolytic effect of each strain was measured by comparing it to the reference virus 17+. The reference virus used for comparisons, 17+, has been highlighted with a grey background.

2.2 Sensitivity to antiviral treatments

2.2.1 Acyclovir sensitivity

Sensitivity to acyclovir was determined for each strain with a plaque assay conducted in Vero cells. The acyclovir-treated cells, with concentrations ranging from 0.03125 μg/ml to 128 μg/ml, were infected with 50 PFU of each virus in a 96-well format. Human IgG was added to the cells 1 hour after the infection to prevent the formation of any secondary plaques, and the plaques were calculated at 72 hpi. As in previous experiments (Kalke et al., 2020), the limit of acyclovir sensitivity was set at a half-maximal inhibitory concentration (IC_{50}) value of 1.90 μg/ml. The acyclovir-resistant control Δ305 reached and exceeded this level with an IC_{50} value of 2.61 μg/ml. None of the other viral strains could be considered resistant to acyclovir (Fig. 13). The IC_{50} values for the clinical strains ranged from 0.14 μg/ml to 1.13 μg/ml, with the average IC_{50} value being 0.30 μg/ml with a SD of 0.18 μg/ml. V1 possessed the highest IC_{50} value of the clinical strains at approximately 1.13 μg/ml, followed by KOS, V7, and V16 with IC_{50} values of 0.76 μg/ml, 0.65 μg/ml, and 0.65 μg/ml, respectively. In contrast, the clinical strains V10 and V11 were the most susceptible to acyclovir, with an IC_{50} value of 0.14 μg/ml.

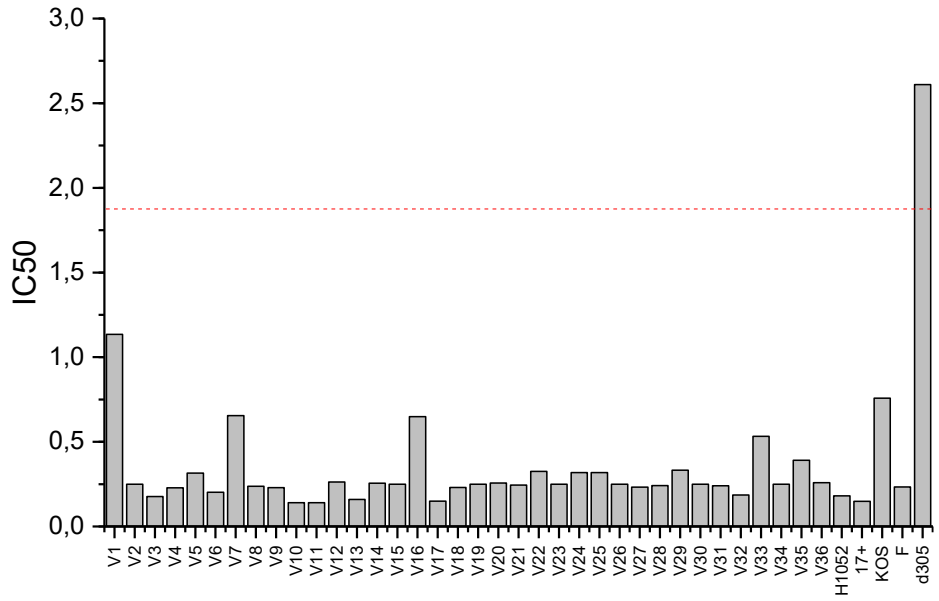

Figure 13. The individual IC_{50}-values of acyclovir for each strain. Sensitivity to acyclovir was measured with an assay conducted in Vero cells, which were first treated with acyclovir concentrations ranging from 0.03125 µg/ml to 128 µg/ml. The cells were then infected with 50 PFU of each virus in a 96-well format. Human IgG was added to the cells 1 hour after the infection to prevent the creation of any secondary plaques. A plaque assay was used to calculate the titers at 72 hpi, which were then utilized to determine the IC_{50}-values for each strain. The bars represent the IC_{50} value of each strain. As in previous research (Kalke et al., 2020), the limit of acyclovir sensitivity was set at an IC_{50}-value of 1.90 µg/ml, marked with a red dashed line on the graph.

2.2.2 Sensitivity to siRNA swarms

Each strain's sensitivity to siRNA swarms was tested by infecting U373MG cells, which had been transfected with siRNA swarms, with 1000 PFU of each of the clinical and reference strains. The released virus was measured from the supernatants of each treatment group after a three-day incubation. Two siRNA swarms were used: the HSV-specific U_L29 and the non-HSV-specific control swarm PET. Both swarms had had their adenosines fully replaced with fluoro-modified adenosines (please see chapter 4.9 for more details on the modification).

Viral replication in wells that had been treated with 100% F-A U_L29 was very low, as only V7, V12, V24, and V27 produced any plaques, with V27's possessing the highest titer at only 125 PFU/ml (Fig. 14). The efficacy of the swarms was 100.00% for most of the clinical strains and all of the reference strains, with only strains V7, V12, V24, and V27 producing any plaques. For these strains, the efficacy of the swarms ranged from 99.78% to 99.97%. In contrast, the virus concentrations in wells treated with 100% F-A PET were 10,000-100,000 -fold higher, and each infection produced high amounts of plaques.

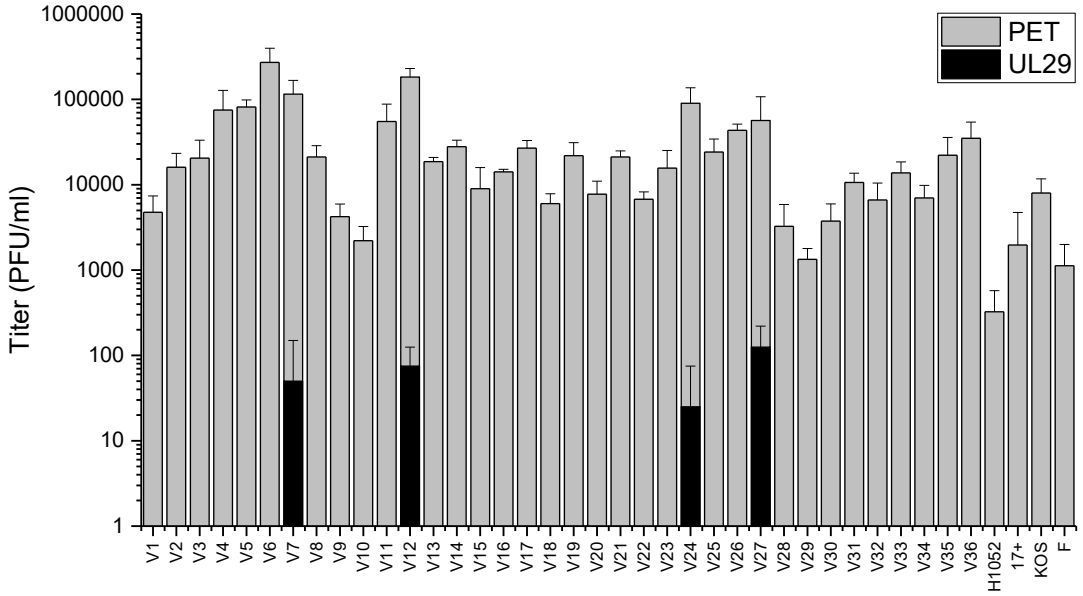

Figure 14. Virus titers of cells treated with either antiviral 100% F-A U_L29 or control swarm 100% F-A PET. Each strain's sensitivity toward siRNA swarms was tested by infecting U373MG cells transfected with siRNA-swarms with 1000 PFU of each of the clinical and reference strains. The titer of each virus was measured after a three-day incubation by utilizing a plaque assay. The bars represent the measured titer for each strain, with the black representing cells treated with 100% F-A U_L29 and grey bars representing cells treated with 100% F-A PET. The whiskers depict the standard deviation of the mean (N=4 per treatment). The titers are represented with a logarithmic scale.

2.3 Viral genetics

2.3.1 Eastern/Western clades

The clade of each virus isolate was determined with PCR by using primers that can detect the minor genetic variance between the clades (Lasanen et al., 2021). According to the results, most of the strains represent the Eastern clade (Table 4). Reference strains 17+, F and KOS were used to ensure the veracity of the result, and each was located in the expected clade. As H1052 is based on the reference strain 17+, it localized in the same clade (Mattila et al., 2015).

Table 4. Strains of interest arranged by their clade. The clade of each virus isolate was determined with by using PCR primers that detect the minor genetic variance between the clades (Lasanen et al., 2021). H1052 was placed in the Western clade, as it is based on the reference virus 17+.

Western clade	Eastern clade
V4	V1
V5	V2
V10	V3
V11	V6
V14	V7
V22	V8
V23	V9
V31	V10
V32	V12
V33	V13
V36	V15
H1052	V16
F	V17
17+	V18
	V19
	V20
	V21
	V24
	V25
	V26
	V27
	V28
	V29
	V30
	V34
	V35
	KOS

2.3.2 Phylogenetic tree of glycoprotein genes

Two viral genes that contribute to cell-to-cell spread, U_L27 and U_S7, were analyzed by first utilizing PCR to amplify the sequences of interest that were then purified and

subjected to sequencing. Phylogenetic trees were then created based on the sequence information received. The strains were placed in the tree based on the similarity in their genetic code, and strains closer to each other contain similar mutations, such as single nucleotide polymorphisms.

When the U_L27 –gene was analyzed, four larger subgroups were formed (Fig. 15). The first group consisted of the clinical strains V6, V22, V3, V31, V26, V30, V17, V24, V16, V13, V2, V27, V29, V20 and KOS, while the second group consisted of clinical strains V15, V18, V19, V32, V21, V28, V34, and V35. The last two groups, formed around the reference strains F and 17+ were smaller: clinical strains V4, V23, and V14 showed close resemblance to F, and the strains V25 and H1052 were placed close to 17+.

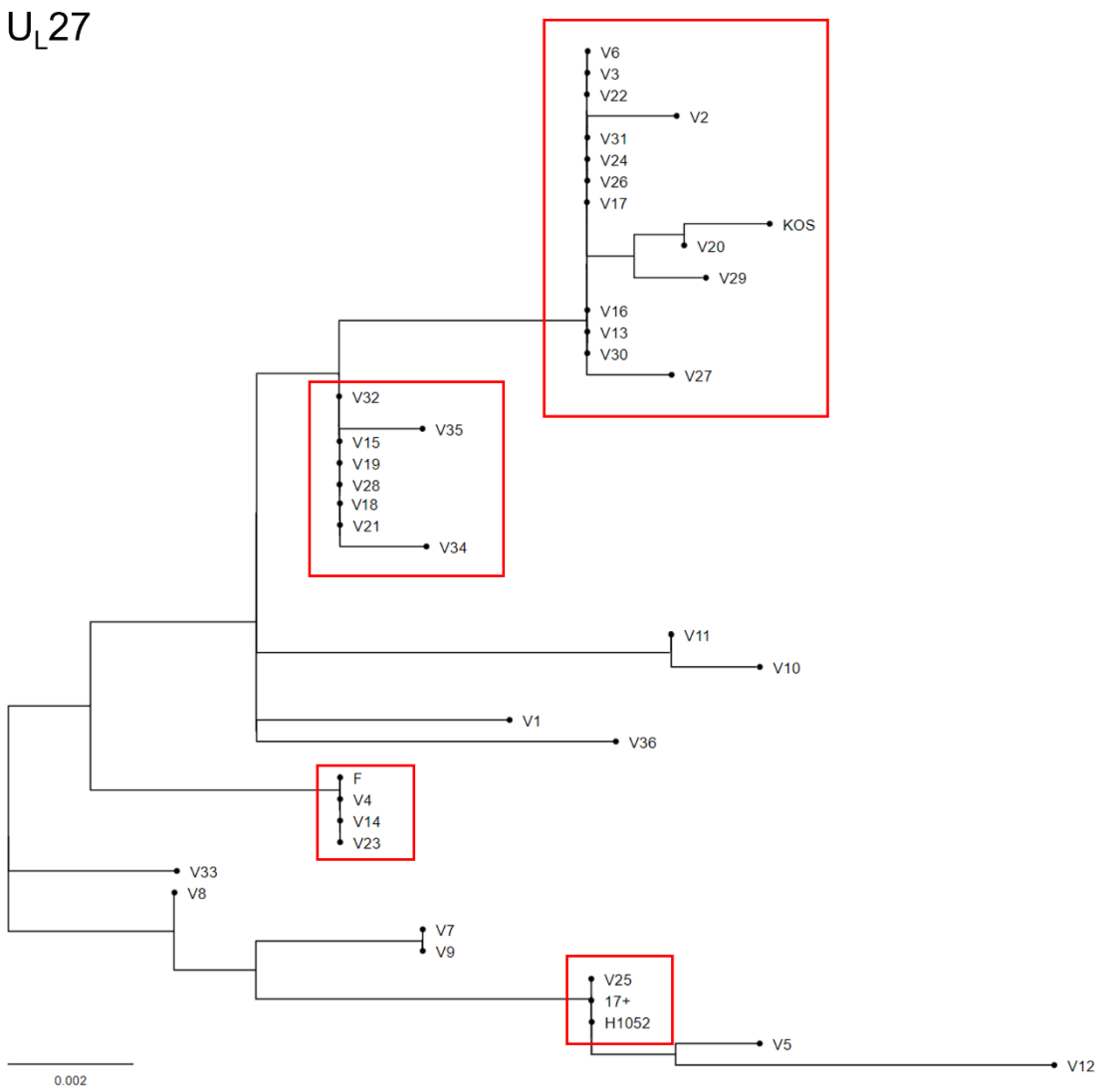

Figure 15. Phylogenetic tree based on sequencing information from U_L27-gene. The phylogenetic tree was built based on information from the targeted sequencing of the gene of interest. The groups have been specified with red boxes.

Three groups were formed upon the analysis of the U_S7 –gene (Fig. 16). The first group was by far the largest, consisting of the clinical strains V7, V9, V1, V28, V24, V29, V31, V26, V19, V12, V15, V6, V17, V35, V21, V16, V18, V30, V34, V2, V13, V3, V8, and V20, and the reference strain KOS. The second major groups consisted of the reference strain F and the clinical strains V14, V22, V23, V4, and V33 and the final third group was made up of the reference strains 17+ and H1052 with clinical strains V25 and V27.

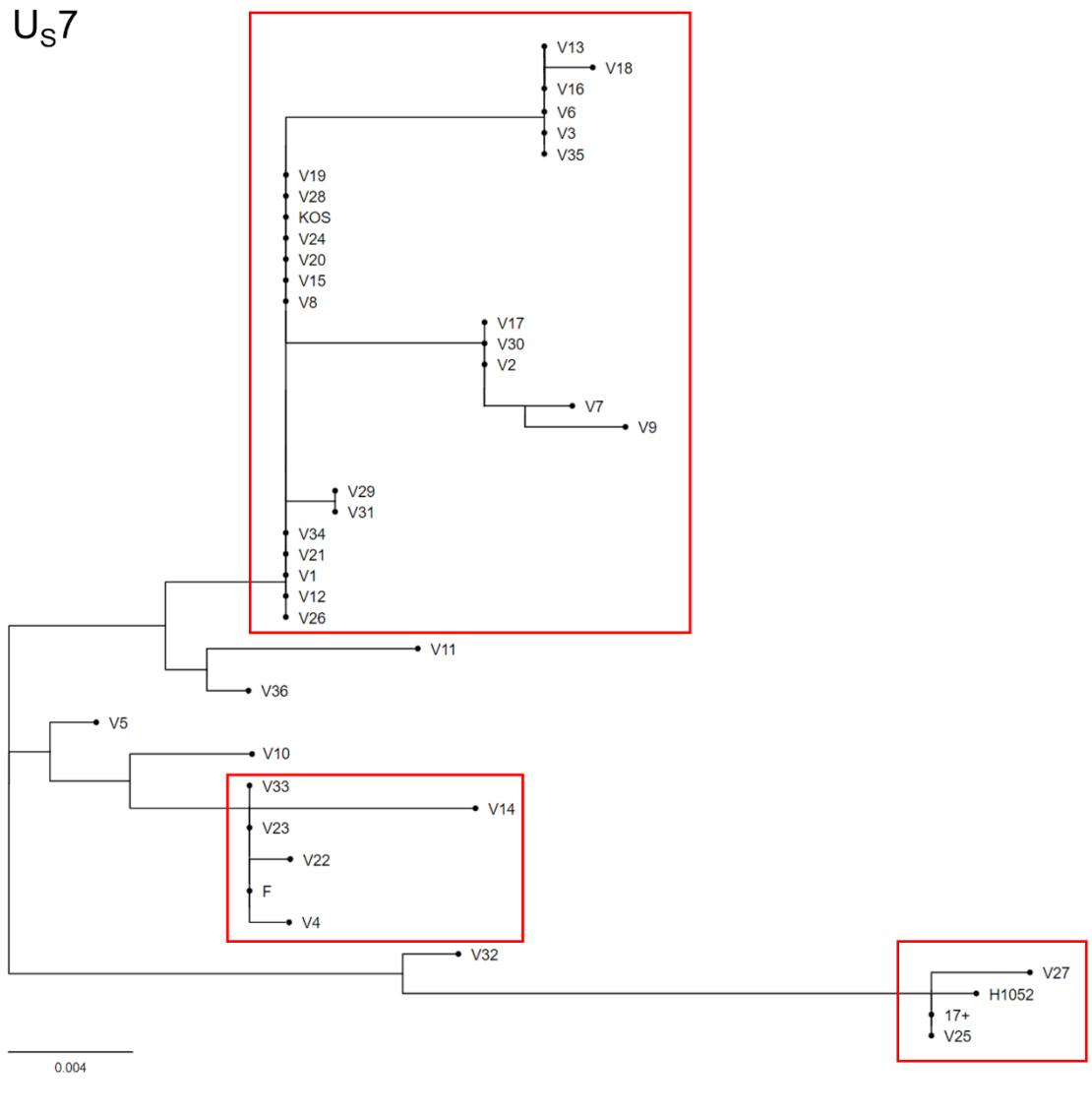

Figure 16. Phylogenetic tree based on sequencing information from U_S7-gene. The phylogenetic tree was built based on information from the targeted sequencing of the gene of interest. The groups have been marked with red boxes.

3. Discussion

3.1. Summary of the study

The aim of this Master's thesis was to characterize a selection of 36 clinical strains with regard to their potential as gene vector development candidates. This required the evaluation of multiple different properties each strain possessed, ranging from infectivity to responsiveness to pharmacological treatment. All available confirmed HSV-1 -strains were utilized in the experiment. First, the replication characteristics of each strain were studied in four cell lines to determine both the shed virus titer from the supernatant and the cell bound virus titer from lysed cells. Second, the oncolytic potential of each strain was assessed in three cancer cell lines. Third, a growth curve was created for all the clinical strains to determine their growth rates. A second, smaller growth curve was obtained in a repeat experiment, with the strain selection based on preliminary results from the cell bound and shed titer analyses. Fourth, the strains' response to treatment with the nucleoside analogue acyclovir and siRNA swarms was determined. Finally, a genetic analysis of three relevant glycoproteins was performed on every clinical strain. Analysis of one of the glycoproteins was conducted to detect each strain's clade on the eastern-western axis, while the other analysis was related to glycoprotein sequences involved with the viral particle's entry to the cell.

3.2. The strains displayed a wide variety of infection profiles

The reference strains included in the experiment were 17+, KOS, and F. Additionally, H1052, an attenuated vector backbone with a deleted neurovirulence gene and a GFP-marker gene, was used as a control. Of these virus strains, 17+ was used as the point of comparison in most of the experiments, as the infection characteristics of the strain are well known and it has been used as a reference strain in previous experiments (Bowen et al., 2019). In general, the results with the other reference viruses didn't greatly differ from 17+, with the exception of the H1052, which had significantly less cell bound virus in three of the four cells lines. This was expected, as H1052 has been modified with the attenuation of both copies of its neurovirulence gene (Mattila et al., 2015), which has a major repressive effect on its replication ability. Curiously, though this effect is not supposed to be as pronounced in cancer cell lines (Liu et al., 2003), the strain displayed the lowest titers of all the strains in both U373MG and WM1799 cells. While there is

proof that the deletion of the $\gamma_1$34.5 gene attenuates the replication of the virus in glioblastoma cells (Kanai et al., 2012), no such reactions seem to have been observed in WM1799 human melanoma cells, or melanoma cells in general. On the contrary, experiments with $\gamma_1$34.5-deleted vectors such as HSV 1716 have shown promising results with this particular type of cancer (MacKie et al., 2001). This suggests that H1052 may harbor yet unknown secondary mutations that affect cell-to-cell spread, which is quite possible as H1052's entire sequence is yet to be established.

Viruses have two patterns of spreading: they can either be shed from their host cells and then infect other cells externally, or be directly transmitted from cell to cell (Zhong et al., 2013), with the latter pattern observed as typical for HSV (Abaitua et al., 2013). There were no instances of a strain possessing a low cell bound virus titer and a high released virus titer, which suggests that all clinical strains that possessed an apparently low cell bound titer also had a low overall infectivity (Fig. 6). As low infectivity is generally a detrimental property for oncolytic vector prospects due to the issues it causes with production and, indeed, the spread of virus in the target cells, it may be reasonable to view these strains as less viable for oncolytic vector development. However, a less aggressive infection could also be viewed as a favorable property for other types of vectors, such as vaccine viruses. Many such clinical strains could be identified. V2 proved especially notable, as it produced significantly less shed virus than the reference in three cell lines. Other similar clinical strains possessing significantly lower shed titers than 17+ in more than one cell line included V18, V30, V32, and V33. Expectedly, none of these strains displayed a significantly higher cell bound titer than 17+ in any of the cell lines. However, V33 was the only one to have a significantly lower cell bound titer than the reference, which occurred in Vero cells.

On the other hand, strains V4, V5, V7, V12, and V36 had a significantly higher amount of cell bound virus than 17+ in the cell lines U373MG and HCE. V4, V5, and V12 also possessed a significantly higher shed titer than 17+ in the same cell lines and V7 in a single cell line, and as such, it is likely that these strains have an overall high infectivity. V36, however, did not possess a significantly different shed titer in any of the cell lines. Indeed, in HCE cells, the proportion of shed to cell bound virus for this strain was significantly lower than with the reference virus, a trait it shared with the clinical strain V29. This suggests that V36 and perhaps V29 have a higher tendency to stay cell bound than the other clinical strains. Having a low proportional shed titer could be seen as a favorable property for a viral vector, as cell-to-cell spread is the more effective pattern of

proliferation, owing in part to the fact that extracellular viruses are far more vulnerable to the antibody-mediated immune responses of the host. Additionally, having a low shedding viral vector could help minimize the risk of the vector unintendedly spreading to the environment through excretions (Schenk-Braat et al., 2007).

While strains V9, V12, V13, V19, V20, V21, and V26 also had a significantly lower shed to cell bound ratio than the reference viruses, these results were observed in Vero cells, where the overall released virus level was notably lower than in other cell lines (Fig. 6). Indeed, V12 had a higher shed to cell bound ratio in U373MG cells, which suggests that the host cell line can have a major impact of the infectivity of the clinical strains. In Vero cells there were only two instances of a clinical strain possessing a significantly higher shed or cell bound titer than the reference virus. The seemingly poor infectivity of the clinical strains in comparison to the reference strains in Vero cells could be explained by the fact that the reference strains had already undergone multiple passages in the cell line previously, which has proved to increase the infection efficiency of viruses (Kuny et al., 2020). In U373MG cells none of the strains had a significantly lower titer than 17+ and the same was mostly true of HCE cells, though there were three instances of a significantly lower shed titer than the reference virus. WM1799 melanoma cells, however, were unique in that no statistically significant differences could be observed between the cell bound titers. It is difficult to ascertain the reason behind this phenomenon, though it can be speculated that none of the strains possessed any mutations that would have rendered them either more infectious or less infectious in the cell line. Considering these results, and seeing as WM1799 is a cancer cell line, it would have been beneficial to conduct a viability assay with the cell line alongside the others. Unfortunately, this was not possible in the scope of this thesis.

3.3. All clinical strains proved oncolytic across tested cell lines

The oncolytic potential of each strain was measured in three cancerous cell lines, each representing a different type of cancer. Regardless of the cell line, the clinical strains proved oncolytic, and a drop in viability could be observed in every cell line for every strain (Fig. 12).

The oncolytic effect was at its strongest in Raji cells, a B-lymphoma cell line in which cellular viability after infection ranged from 7% to 34%. Hematopoietic cells are resistant to HSV-1 replication *in vivo* (Wu et al., 2001), and accordingly, oncolytic HSV (oHSV)

have seen only very limited testing in such cell lines. Nevertheless, a study conducted with a third-generation oHSV in Raji cells demonstrated a significant drop in viability compared to the mock treatment (Ishino et al., 2021), roughly in line with our own discoveries. Moreover, studies utilizing an oncolytic reovirus (Alain et al., 2002) and vaccinia virus (Lei et al., 2022) have recorded a similar drop in viability to what was observed here.

Though the oncolytic effect of the clinical strains was not as severe in the SW480 adenocarcinoma cell line, it was still notable, with the viability of the infected cells ranging from 17% to 43%. As in a previous experiment conducted with HSV and SW480 cells, the clinical isolates or "wild types", proved to be the most oncolytic with very similar drops in viability observed (Haghighi-Najafabadi et al., 2021). Comparing these results with ones obtained from experiments where a recombinant measles virus (Amagai et al., 2016) and an adenovirus (Gao et al., 2019) had been used against the cell line, it could be suggested that HSV seem to have more oncolytic potential than other viruses. However, it should be noted that these results might not be directly translatable owing to the different aims and design of these experiments.

The effect was the least pronounced in U373MG cells, where cellular viability after infections ranged from 60% to 87%. Similar results were observed in a previous experiment where an oncolytic adenovirus was used against multiple glioma cells lines, as the virus was unable to cause a major drop in viability in this cell line with low MOI infections even 5 days after infection (Vera et al., 2016). Even the modified oHSV G47Δ had an effect comparable to our clinical strains on the cellular viability after three days (Sgubin et al., 2012). As such, the poor effectiveness the clinical strains displayed against this cell line should not be considered a poor result, but rather it suggests that several modifications would be needed for oHSV targeted toward gliomas.

Regardless of the cell line, however, the drop in viability caused by the clinical strains was relatively uniform for the clinical strains, though not for the reference strains. Previously, it has been demonstrated that fresh clinical isolates have more oncolytic potential than reference strains like 17+ (Liu et al., 2003). Indeed, the passages of our isolates were low, ranging from 2 to 4.

Nevertheless, some strains proved more oncolytic that others. Of all the clinical strains, V35 showed the most oncolytic potential, as its effect was significantly stronger than 17+ in all three cell lines. Other notably oncolytic strains included V2, V4, V5, V27, V29, and V31, all of which proved significantly more oncolytic than the reference virus in two

separate cell lines. At the other end of the spectrum, V17 caused a significantly smaller drop in viability when compared to 17+ in U373MG cells, though its oncolytic potential did not significantly differ from 17+ in other cell lines. Similarly, V10 proved significantly less oncolytic than 17+ in both U373MG and Raji cells, though it also caused a significantly larger drop in viability in SW480 cells. Thus V10 and V17 are the least suitable candidates for continued oncolytic virus development, though their low oncolytic potential could also be considered a positive property for other gene therapy applications.

3.4. The growth rates for all strains were relatively uniform

The growth curves for most of the clinical strains proved to be quite similar, with an expected, clear drop seen at 6 hpi and the highest virus concentration achieved in the supernatant at around 48 hpi, after which the titer started to drop for most strains (Fig. 8). This type of growth profile is typical for HSV-1 (Leege et al., 2009; Li et al., 2011), however, there were also multiple strains that had differing results, most notably V2 and V11 which both showed a considerably later increase in titer, occurring between the 48 hpi and 72 hpi time points. This could be interpreted as the strains having a slower infection progression, and this theory is supported by the fact that V2's titer was the lowest out of any strain at 48 hpi while V11's was the third lowest (Fig. 8). However, it should be noted that the shed virus titer of V2 was already observed to be significantly lower than 17+'s in Vero cells, suggesting that the strains infection might not be very productive. V11 produced no such results. It is also worth noting that all of the reference strains displayed similar properties to these two clinical strains, insofar that all of them experienced a dramatic drop in titer at the 6 hpi time point, followed by relatively slow increase in titer, which reached its highest value at 72 hpi.

The fact that none of the clinical strains could reach the initial infection titer was surprising, as most of the strains in the second growth curve experiment reached and exceeded the initial infection titer (Fig. 9), suggesting that this result may be derived from the employed methodology. Indeed, reaching the desired 5 MOI with the available virus stocks proved difficult, and necessitated the limiting of the cell count in the wells. It is possible that this resulted in some of the viruses being unable to find cells to infect, and as such, a large part of the infectious material could not reach a cell and was subsequently removed during the wash prior to 6 hpi time point. This hypothesis is further supported by the fact that the more successful second growth curve was conducted with a higher

cell confluency. However, as stock propagation is a time-consuming process and can subject the viral stocks to cross-contamination, it proved unfeasible to repeat the process for all of the strains within the constraints of this thesis.

In general, the growth curves of each clinical strain were mostly uniform, with the variance of titers at the different time points possibly stemming from the difference in the initial infection titer and cell confluency. Strains V2 and V11 proved to cause a slower infection that the other strains in the first growth curve experiment, though in the second experiment this was no longer the case for V11, while V2 was not utilized. Nevertheless, all of the strains proved capable of replicating in Vero cells, and as such, none can be considered unsuitable for vector development or production based on their growth characteristics.

3.5. All clinical strains are responsive to both acyclovir and RNAi treatment

Sensitivity to acyclovir was determined for each HSV strain, and though some strains displayed notably higher IC_{50} than others, it was found that none of the values exceeded the level considered to represent acyclovir resistance (Fig. 13). This is an unsurprising finding, as the prevalence of acyclovir resistant strains is very low among the population, ranging from 0.3% in immunocompetent individuals to 7% in immunocompromised individuals (Bacon et al., 2003). This result is also in line with previous experiments (Bowen et al., 2019; Kalke et al., 2020), whereby none of the tested clinical strains proved resistant to acyclovir treatment. Nevertheless, this is an important discovery, as acyclovir and other nucleoside analogues can be used as an “emergency brake” to cease the proliferation in the highly unlikely event that the HSV-based vector should start multiplying out of control.

Another, more novel method of stopping the spread of HSV is based on the utilization of RNAi through the use of siRNA swarms. Previously, this form of treatment has been proven efficient against all tested strains of the virus, even those that were known to be acyclovir resistant (Kalke et al., 2020). As expected, similar results were obtained during the testing of the clinical strains in this experiment (Fig. 14), with all but four of the clinical strains completely unable to produce any new viruses when the cells had been treated with siRNA. Even in cases where viruses could be detected, the measured titer was significantly lower than in cells treated with the non-specific swarm PET, further

reinforcing the hypothesis that HSV-specific RNAi treatment is an effective alternative treatment *in vitro*.

3.6. Each strain could be assigned with an Eastern or Western genetic trait

By utilizing methodology developed by Lasanen et al. (2021), all of the clinical strains could be located in the Eastern and Western clades typical of Finnish herpesviruses, with around two thirds of the viruses belonging to the Eastern clade (Table 4). This is in line with previous results (Bowen et al., 2019), indicating that the eastern variant of the virus may be more ubiquitous in Finland. However, as the anonymized clinical samples were chosen based on availability, and the demographic data of the original donors is unavailable, more profound analysis of the results is currently impossible.

3.7. Circulating variations in U_L27 and U_S7 have only limited effect on replication properties

Phylogenetic trees based on the genes U_L27 and U_S7 indicated that multiple distinct subgroups could be observed (Fig. 15, 16). However, when these groups were cross-referenced with results from other experiments within the thesis, it could be concluded that the mutations in these genes had no discernible impact on the other characteristics of the viruses in question.

When comparing the results of the shed- to cell bound experiment to the phylogenetic trees, only very inconsistent parallels could be drawn, which is in agreement with previous observations (Bowen et al., 2019). The reference virus F and the clinical strains closely related to it seemed to have a lower shed titer but also a higher proportion of shed virus overall in Vero cells, though the effects are not statistically significant. Furthermore, V18, V19, V21, and V32 were closely related in terms of the U_L27 gene, but also had closely matched cell bound titers. However, as none of the cell bound titers were significantly different from 17+, these observed genetic differences appear to have no effect on the strains' infectivity. The mutations observed in this limited set of sequences seemed to have no detectable effect on the oncolytic efficiency in any of the cell lines, either, and similarly, no correlations could be found between the pharmacological sensitivities of the strains and their genetics. As such, it can be theorized that the change in genotype brought on by these mutations does not affect the phenotype of the infections caused by the viruses (Bowen et al., 2019).

Perhaps the clearest differences, however, could be observed when comparing Eastern and Western strains. Especially in U_S7 sequence -based results, the groups consisted mainly of either Western or Eastern strains, with the exception of the reference strain 17+'s group, which contained both kinds of strains. Considering the evolutionary aspect of the strains, this is not surprising, yet it lends credibility to the results of the clade analysis.

3.8. Conclusions and future considerations

Overall, several notable strains could be distinguished based on their characteristics (Table 5). One of the most important properties a viral strain can have on a base level is high replication capacity. Strains V4, V5, V7, V12, and V36 proved particularly noteworthy in this regard. Furthermore, of these strains, V4 and V5 proved to have notably higher oncolytic potential when compared to the reference virus, making them the first candidates for further developments in creating an oHSV. However, two strains, V10 and V17, had significantly less oncolytic potential than the reference. Though more experiments in cell lines representing other target tissues would be required to confirm this, these less cytotoxic vectors could be considered interesting development targets for applications such as rescue vectors.

Perhaps the most unique strain to have emerged, however, is V2. Its shed virus titer was lower than the reference across all four cell lines, suggesting that it, like V29 and V36, has a strong bias toward spreading cell-to-cell. During the experiment, its cell bound titer did not significantly differ from the reference virus' results. However, when considering the results from the growth curve experiment, it needs to be taken into account that this might have been due to the slow growth rate of V2. Most intriguing, however, was the fact that V2 also proved to be significantly more oncolytic than 17+ in SW480 cells and Raji cells, meaning that it has a high cytotoxic potential as well as high cell bound component, both of which could be considered attractive attributes for an oHSV.

However, it should be noted that any results presented in this thesis should be considered preliminary, owing to the general nature of the experiments performed herein. Additional experiment in other cell lines will need to be conducted in various different cell lines to gain a more thorough understanding of the clinical strains. Nevertheless, the results of this thesis suggest that many of the 36 clinical strains showed potential for continued development as oHSV, and in some cases, for other applications. The baseline

information compiled in this thesis could serve as a useful baseline to base future decisions on.

Table 5. Notable clinical strains.

Clinical strains	Notable characteristic	Characteristic observed during
V4, V5, V7, V12, V36	High infectivity	Determination of cell bound and shed virus concentrations
V29, V36	High proportional cell bound titer	
V2, V4, V5, V27, V29, V31, V35	High oncolytic potential	Determination of cytotoxicity in cancerous cells
V10, V17	Low oncolytic potential	
V2	Slow infection progression	Growth curve

4. Materials and methods

4.1 Cell lines

Multiple cell lines were utilized during the experiment (Table 6). An African green monkey kidney cell line referred to as Vero (CCL-81, ATCC, Manassas, VA, USA) was used for all plaque assays as well as the acyclovir assay and for the growth curve. A human glioblastoma cell line, currently reclassified as U251 (HTB-17, ATCC) but here referred to as U373MG for the sake of continuity with earlier publications from the group, was used for the RNA interference experiment. Furthermore, these Vero and U373MG cells were used to determine the replication properties of the clinical strains alongside a human corneal epithelial cell line referred to as HCE (kindly provided by Arto Urtti from the University of Helsinki and the University of Eastern Finland), and a metastatic human melanoma cell line referred to as WM1799 (WM1799-01-0005, Rockland, Limerick, PA, USA). Additionally, the oncolytic potency of the strains was characterized using U373MG cells, a Burkitt's lymphoma cell line referred to as Raji (CCL-86, ATCC), and a human colon adenocarcinoma cell line referred to as SW480 (CCL-228, ATCC).

Table 6. Cell lines used in the experiments.

Cell line	Origin	Maintenance
Vero	African green monkey kidney cells	Medium 199 or DMEM with 7% FBS and gentamycin (20 µl/100 ml)
U373MG	Human glioblastoma cells	DMEM with 7% inactivated FBS, gentamycin (20 µl/100 ml), and 1% GlutaMAX
HCE	Human corneal epithelial cells	DMEM with 7% inactivated FBS, gentamycin (20 µl/100 ml), and 1% GlutaMAX
WM1799	Metastatic human melanoma cells	MCDB 153 (80%) with 20% Leibovitz's L-15, 2% FBS, and $CaCl_2$
Raji	Lymphoblast-like cells from a Burkitt's lymphoma	RPMI 1640 with 10% inactivated FBS, gentamycin (20 µl/100 ml), and 1% GlutaMAX
SW480	Human colon adenocarcinoma cells	DMEM with 7% inactivated FBS, gentamycin (20 µl/100 ml), and 1% GlutaMAX

Vero cells were maintained in M199 medium (Lonza, Basel, Switzerland) with 7% heat-inactivated FBS (Gibco, Carlsbad, CA, USA). U373MG, HCE, and SW480 cells were maintained in DMEM with Hepes (Lonza) that had been supplemented with 7% FBS and

1% GlutaMax (Gibco). Finally, Raji cells were maintained in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 1% GlutaMax. All cell lines were cultured with the antibiotic gentamycin. The upkeep of WM1799 cells was handled off-site by collaborators, according to the manufacturer's instructions, utilizing a medium with 80% of MCDB 153 (Sigma Aldrich, St Louis, MO, USA), 20% Leibovitz's L-15 (Sigma Aldrich), 2% FBS (Rockland), and CaCl2 (Sigma Aldrich). A 1:10 dilution of 0.5% Trypsin-EDTA (Gibco) in PBS (Gibco) was used to detach the cells during cell passaging.

4.2 Viruses

This project includes genetically modified viruses and is included in a larger research project under the permit 018/M/2018, which has been obtained from the National board of gene technology. Approval for the study of anonymous HSV isolates has been provided by the Turku University Central Hospital under the permit number J10/17. The clinical isolates used in this experiment were originally obtained from anonymous clinical samples of herpetic lesions and archived by the diagnostic service of the Department of Virology, University of Turku. However, all viruses used in the project have been obtained from viral cultures in non-human primate cell lines, and as such, do not have a human origin. The samples represent the current circulating strains of HSV-1 in Finland, though the effects of recent immigration are not represented in the samples. Upon sampling, an immunoperoxidase rapid culture assay had been used to type the viruses as HSV-1 (Ziegler et al., 1988), which was then further confirmed by an HSV type-specific gD (U_S6) gene-based PCR test. Prior to the beginning of the experiment, the anonymous isolates were randomized, after which they were referred to as strains V1-V36. Furthermore, the green fluorescent protein-expressing strain H1052, which has had its neurovirulence gene deleted ($\Delta\gamma_1$34.5) (Mattila et al., 2015), was also included as a control.

Multiple reference wild-type strains were used: HSV-1 F (Ejercito et al., 1968), HSV-1 17+ (McGeoch et al., 1986), and HSV-1 KOS (Smith, 1964). Furthermore, an acyclovir-resistant, thymidine kinase -deficient strain Δ305 (Post et al., 1981), was utilized as a control in the acyclovir assay. Viral stocks for the assays were prepared prior to commencing the study by first infecting fully confluent Vero T-25 flasks (Sarstedt, Nümbrecht, Germany) with 0.1 PFU per cell of each virus and then incubating them at

37 °C, 5% CO_2 until the cytopathic effect reached its plateau. Autoclaved 9% milk (Valio, Helsinki, Finland) in PBS was then added to the flasks, which were placed at –80 °C to detach the cells. The contents were thawed again and aliquoted into cryovials (Corning Inc., Corning, NY, USA), which underwent three freeze-and-thaw cycles. Afterwards, the aliquots of each virus were resuspended and aliquoted again. The titers of the reference strains had to be determined with a plaque assay, whereas the titers of the clinical strains and the gene-modified viruses were already known prior to the start of the experiment (Table 7).

Table 7. The viruses used in the experiment and their titers.

Virus	Titer (PFU/ml)	Virus	Titer (PFU/ml)
V1	1,18E+06	V22	2,00E+06
V2	3,07E+05	V23	2,07E+06
V3	1,19E+06	V24	2,80E+06
V4	7,55E+05	V25	2,10E+06
V5	4,00E+06	V26	9,65E+05
V6	1,50E+06	V27	1,50E+06
V7	1,00E+06	V28	3,00E+07
V8	2,07E+06	V29	1,15E+07
V9	2,20E+06	V30	2,35E+06
V10	4,45E+06	V31	1,06E+07
V11	1,87E+06	V32	6,35E+06
V12	4,00E+05	V33	5,95E+06
V13	1,10E+06	V34	2,10E+06
V14	1,50E+07	V35	7,65E+05
V15	8,25E+06	V36	2,90E+07
V16	3,20E+06	H1052	4,90E+05
V17	3,70E+05	17+	7,00E+06
V18	2,45E+06	KOS	3,20E+05
V19	7,45E+05	F	1,00E+07
V20	3,85E+05	Δ305	3,50E+09
V21	9,00E+05		

4.3 Plaque assay

A plaque assay was utilized to measure the titer of the viruses throughout the study. First, a set of dilutions was prepared on a 96-well plate (Corning Inc.) with fully confluent Vero cells in 100 µl of culture medium. 11 µl of supernatant was transferred to the first row of the titration plate and mixed. The rest of the dilutions were prepared by always transferring 11 µl to the next row. The plates were then incubated for approximately 1-2

h at 37 °C and 5% CO_2, after which 100 µl of DMEM (incl. Hepes) supplemented with 2-5% FBS, 20 µl/100 ml of gentamycin, and 80 mg/l of human IgG HyQvia (Takeda, Tokyo, Japan). Some experiments necessitated the use of a 24-well plate (Corning Inc.) instead of a 96-well plate. In such cases, the wells contained 900 µl of medium, and 100 µl of supernatant was transferred from well to well, and 400 µl of the IgG-containing medium was added. The plates were then incubated at 37 °C and 5% CO_2 for approximately 72 h, after which the cells were fixed with 4 °C methanol and stained with 0.1% crystal violet. The number of plaques was then counted and used to calculate the titer as PFU/ml units for each virus.

4.4 Acyclovir assay

Sensitivity to acyclovir was analyzed in a 96-well format for each strain, as previously described in Bowen et al. (2019) and in Kalke et al. (2020), with HSV-1 Δ305 as an acyclovir-resistant control. Fully confluent Vero cells were pre-treated with varying concentrations of acyclovir (Sigma Aldrich), ranging from 128 µg/ml to 0.03125 µg/ml. These wells, alongside control wells without acyclovir, were then infected with 50 PFU of each virus in duplicates by adding infectious medium (DMEM with 5% FBS and gentamycin) on top of the ACV-supplemented medium. After a 1-3 h incubation period, medium with human IgG (DMEM incl. Hepes supplemented with 2% FBS, 20 µl/100 ml of gentamycin, and 80 mg/l of IgG HyQvia) was added to the cells to prevent any secondary plaque formation. After a 72-hour incubation at 37 °C and 5% CO_2, the cells were fixed with 4 °C methanol and then stained with crystal violet as with the plaque assay. The reduction in the number of plaques was utilized to calculate the IC_{50} value of acyclovir for each of the strains.

4.5 Comparison of cell-bound and released virus

To determine the characteristics of viral spread in cell types representing potential target tissues of HSV-1 based gene therapy, methodology previously described in Bowen et al. (2019) was utilized. The experiment was carried out in multiple cell lines: Vero, HCE, U373MG, and WM1799. Fully confluent cells on 96-well plates were infected with 0.1 MOI of each virus in 50 µl of medium (DMEM incl. Hepes supplemented with 2-5% inactivated FBS and gentamycin). In both repeats of this experiment, four parallel replicates represented each viral strain.

After an incubation period of 1.5 h, the infection medium was removed, and the cells washed before being left in 200 µl of fresh culture medium. The cells were then incubated at 37 °C and 5% CO_2. At 24 hpi, the supernatant was collected, and the cells were preserved in 100 µl of 9% autoclaved milk in PBS or in 100 µl of 10% FBS. All samples were stored at –80 °C. Three freeze-and-thaw cycles were performed to break up the cells and to release the virus particles prior to their titrations, while the supernatant samples required no further actions. Finally, a plaque assay was used separately on supernatant samples and cell samples to determine the concentrations of shed virus and cell-bound virus.

4.6 Oncolysis assay

The capacity of the viruses to lyse cancer cells was determined with an oncolysis assay. Three cancer cell lines, U373MG, Raji, and SW480 were infected with 2 MOI of each viral strain in a 96-well format with four parallel samples for each strain. For U373MG and SW480 cells, the infection was carried out by replacing the upkeep medium with 100 µl of viral dilution in DMEM (incl. Hepes) supplemented with 2% FBS and gentamycin. Raji-cells, which are a suspension cell line, were seeded in 50 µl of RPMI 1640 supplemented with 10% FBS, gentamycin, and 1% GlutaMax, and infected with 50 µl of viral dilution made with the same medium. “Cells only” –wells were not infected, so as to act as controls for the experiment. After an incubation period of approximately 1 hour at 37 °C and 5% CO_2, U373MG and SW480 cells were washed twice with medium (DMEM incl. Hepes supplemented with 2% FBS and gentamycin), then left with 100 µl of culture medium.

The viability of the cells was analyzed at 96 hpi with the CellTiter-Glo® viability assay (Promega, Madison, WI, USA). First, the CellTiter-Glo® reagent was added onto cells: 100 µl was added for Raji-cells, but for U373MG and SW480 cells 50 µl of the medium was replaced with the reagent. The plates were then protected from light and mixed for 2 minutes on an orbital shaker, followed by a 15-minute incubation at RT. Finally, VICTOR Nivo Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA) was used to measure the amount of luminescence in each well, which correlates with the amount of ATP and can be used to determine the proportion of viable cells.

4.7 Growth curve

A screening growth curve was composed for each virus by utilizing a modified version of the protocol presented in Nygårdas et al. (2013). 50% confluent Vero cells on a 96-well plate were infected with roughly 5 MOI of each virus in four replicates. After 2 h of incubation at 37 °C and 5% CO_2, the cells were washed and left to incubate under the same conditions with 200 µl of DMEM (incl. Hepes) supplemented with 2% inactivated FBS and gentamycin. 20 µl supernatant samples were taken at 6 h, 24 h, 48 h, and 72 hpi. The titer of each virus at each of these time points was determined with a plaque assay.

Table 8. The strains used in the second growth curve and the reasoning for their inclusion.

Strain	Reason for inclusion
V3	High replication (cell bound titer)
V4	High replication (cell bound titer)
V5	High replication (cell bound titer)
V6	High replication (cell bound titer)
V7	High oncolytic potency
V11	High replication (cell bound titer)
V12	High replication (shed titer)
V16	High replication (cell bound titer)
V22	High replication (cell bound titer)
V27	High oncolytic potency
17+	Reference strain
H1052	Gene modified strain (representative backbone of oHSVs)

The second growth curve was done with a smaller group of viruses. This group (Table 8) was chosen based on the results from earlier experiments. Fully confluent Vero cells on a 24-well plate were infected with approximately 5 MOI of each virus in duplicates. The plates were then incubated at 37 °C and 5% CO_2 for 2 hours, after which the cells were washed with DMEM (incl. Hepes) supplemented with 2% inactivated FBS and gentamycin and then placed back in the incubator with 1000 µl of the same medium. 50 µl samples of the supernatant were taken at the same time points as with the screening experiment, and the titers for each strain were determined with the plaque assay.

4.8 Viral genetics

Quantitative PCR was used to prepare samples for genetic analysis of glycoproteins relevant to cell-to-cell spread, as well as to determine the genetic clades of each strain. For glycoprotein analysis, the genes U_L27 and U_S7 were chosen as targets for the analysis: U_L27 codes for gB, which is essential in the virus's fusion into cell membranes, while

U_S7 codes for gI which is critical in the lateral spread of nontransformed viruses (Roizman et al., 2013). The samples were diluted in PBS, either 1:10 or 1:100 depending on the virus, and the samples were boiled at 98 °C for 10 minutes and then refrigerated while the standards and PCR -mixes were prepared. The PCR -mixes contained H_2O, SYBR Green (ThermoFisher, Waltham, MA, USA), and different primers based on the experiment. The PCR-mix was added to each of the RotorGene tubes (Qiagen, Hilden, Germany) on a cold block, after which the master mixes were combined with the samples. Additionally, a control series of viral genomes with known copy numbers ranging from 10^8 to 10^1 copies per reaction were prepared for the Eastern/Western genotype determination, of which controls 10^7, 10^5, and 10^3 were utilized during glycoprotein analysis. A "no-template" –control was prepared by adding only PCR-mix and no sample to the tube. The samples were loaded onto the Rotor-Gene Q PCR cycler (Qiagen) and the run (Table 9) was initiated.

Table 9. The PCR run.

Cycle	Cycle point
Hold 1	Hold @ 95°C, 10min 0s
Cycling	Step 1: Hold @ 95°C, 15s
(45 repeats)	Step 2: Hold @ 55°C, 30s
	Step 3: Hold @ 72°C, 45s
Hold 2	Hold @ 95°C, 0min 15s
Melt	Ramp from 72°C to 95°C
	Hold for 90s on the 1st step
	Hold for 5s on next steps

4.8.1 Analytical agarose gel

The primers pairs used in the glycoprotein analysis experiment, U_L27 (5'-CGGTGGTCTCCAGGTTGTTG-3' and 5'-TGGTCTACGACCGAGACGTT-3') and U_S7 (5'-ACGTGTTACGCGTATGGGTC-3' and 5'-TATACCAACAGGGGAGGCGT-

3'), were designed to target U_L27 and U_S7, respectively. After the PCR-run was completed, the integrity of each sample was checked with an agarose gel run. 5 µl of each sample was mixed with 1 µl of DNA Gel Loading Dye (6X) (ThermoFisher) and run in a 1% agarose gel at 40 V for 5 min, and then at 90 V for 1 h. Finally, the DNA in the PCR products was purified with the GeneJET PCR Purification Kit (ThermoFisher) using the provided manufacturer's protocol. The concentration of each sample was measured with a DS-11 spectrophotometer (Denovix Inc., Wilmington, DE, USA), and 5 µl of each sample with both primer pairs were sent for sequencing (LightRun, Eurofins Genomics, Denmark) in 1.5 ml SafeLock Eppendorfs (Sigma Aldrich).

4.8.2 Eastern/Western genotyping of virus strains with PCR

The recognition of the genetic clade was carried out based on sequence variation of the glycoprotein gG-1 gene (Bowen et al., 2019; Lasanen et al., 2021). In addition to the "no-template" –control, a water control was added for the assessment of possible PCR contaminations by substituting the adding H_2O to the PCR-mix. The PCR run was conducted twice: the first run used the primers referred to as S1, which detects Western-type HSV genome, and R, which anneals to all HSV-1 strains, while in the second run S1 was replaced with F, which anneals to all HSV-1 strains. The copy numbers from each virus were then used to determine their clade.

4.9 Sensitivity of the viral strains to modified antiviral siRNA swarm

The prophylactic antiviral efficacy of UL29-targeting siRNA swarms as an antiviral treatment against the strains was quantified by transfecting U373MG cells with HSV specific or nonspecific siRNA swarms and then infecting them with the strains according to previous work by Kalke et al. (2020) and Levanova et al. (2020). A HSV-specific RNA swarm U_L29 (Romanovskaya et al., 2012) and a non-specific swarm PET (Levanova et al., 2020) were used in the experiment. Both had been 100% 2'-fluoro modified (Levanova et al., 2020).

Two RNA-mixes, containing either UL29 or PET with RNAiMAX Lipofectamine (Invitrogen, Carlsbad, CA, USA) and OptiMEM (Gibco) were prepared at a concentration of 5 pmol/well, according to the manufacturer's protocol. Similar mixes were prepared for the controls, which were an unmodified UL29-swarm, immunostimulatory, nonspecific 88bp double-stranded RNA (Jiang et al., 2011), and a mock transfection mix

which contained only lipofectamine and OptiMEM. The mixtures were vortexed and then incubated at RT for approximately 20 minutes with a second vortexing in the middle of the incubation period. The cell plates were washed and then left with 80 µl of OptiMEM, and 20 µl of the appropriate RNA-mix was added to each well in such a way that there were four parallel wells of both UL29 and PET for each virus. Furthermore, a set of four parallel wells were prepared for each of the control transfections, as well as a set of four wells that were not treated in any way. The plates were then placed in the incubator (37 °C, 5% CO_2) for 4 hours.

After the incubation, the transfection medium in each well was replaced with 100 µl of a corresponding viral dilution with 1000 PFU in DMEM (incl. Hepes) supplemented with 2% of FBS and gentamycin. The control wells were infected with the reference strain 17+, as were the four untransfected wells, acting as infection control. The plates were placed in the incubator (37 °C, 5% CO_2) to be incubated for 48 hours, after which the supernatant from each well was transferred to a separate plate that was stored at –80 °C. The plates were then thawed and the samples titered to determine the efficiency of the antiviral RNAi.

4.10 Live cell imaging

In vitro -imaging of the virus plaques during various parts of the experiment was carried out with the EVOS FL Auto imaging system (ThermoFisher) and with the Primovert inverted microscope (CarlZeiss, Oberkochen, Germany).

4.11 Statistical methods

Statistical analyses were performed with SPSS statistics v.25.0.0.1 (IBM, Armonk NY, USA). The statistical significances were calculated with Mann-Whitney's non-parametric U-test by comparing two individual groups at a time, with the threshold of significance set as a P-value of <0.05. The sigmoidal dose-response curves and their associated IC_{50} values were fitted and calculated with Origin 2016 v.b9.2.3.303 (Academic) (OriginLab Corporation, Northampton, MA, USA).

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Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine deaminase
ATP	Adenosine triphosphate
bp	Base pair
CAR T	Chimeric antigen receptor T
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
EMA	European Medicines Agency
FBS	Fetal bovine serum
FDA	Food and Drug Administration
g	Glycoprotein
GFP	Green fluorescent protein
h	Hour
hpi	Hours post-infection
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
ICP34.5	Infected cell protein 34.5
IC_{50}	Half-maximal inhibitory concentration
Ig	Immunoglobulin
ml	Milliliter
mRNA	Messenger ribonucleic acid
MOI	Multiplicity of infection
oHSV	Oncolytic herpes simplex virus
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
p.i.	Post-infection
RNA	Ribonucleic acid

RNAi	Ribonucleic acid interference
RT	Room temperature
SD	Standard deviation
siRNA	Small interfering ribonucleic acid
TK	Thymidine kinase
T-vec	Talimogene laherparepvec

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