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RNA INTERFERENCE AS POTENTIAL ANTIVIRAL TREATMENT AGAINST ENTEROVIRUS 71

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Abstract

Hand-foot-and-mouth disease (HFMD) is quickly becoming a common childhood disease. HFMD is not usually fatal but when infected by Enterovirus 71 (EV71), one of the causative agents of HFMD, this childhood disease can potentially lead to neurological symptoms, complications and even death. As there are currently no effective treatment against EV71, development of an efficient antiviral therapy is important. This study evaluates the use of RNA interference (RNAi) as a potential antiviral strategy against EV71.

RNAi, a pathway founded by Fire A. in *Caenoharbditis elegans*, is currently one of the most researched pathways due to its potential as an antiviral treatment. The pathway in occurs in the cytoplasm, which is suitable for use against EV71 because its replication cycle also takes place in the cytoplasm. Custom-designed small interfering RNAs (siRNAs) of three different lengths and concentrations were transfected into Rhabdomyosarcoma (RD) cells to target the 3D polymerase region of the EV71 genome. The lengths of the siRNAs used were 19mer, 27mer and 29mer, and the concentrations used were 3nM, 5nM and 10nM. The effectiveness of inhibition of EV71 replication was demonstrated by Western blot and plaque assays.

The results proved that RNAi could be used as a potential antiviral therapy against EV71, and that the inhibition of EV71 was dependent on the concentration of siRNA transfected. The different lengths of siRNAs also affected the efficiency of the viral replication. The results revealed that 19mer siRNAs had the weakest inhibitory effects while 29mer had the best inhibitory effects. Using the results from this study, more assays can be done to provide a numerical representation of the inhibitory effects before proceeding to animal testing.

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1. Introduction

HFMD is quickly becoming one of the major childhood diseases which can be potentially fatal if the causative agent is EV71 [Hellen, 1995]. Infecting mainly children, EV71 is now a virus which raises awareness and alarm for many parents due to its ability to cause neurological symptoms [Kuo *et al*., 2002]. There had been many reports of EV71 outbreaks and deaths around the world since the 1970s and more recently in the past 20 years, the Asia-Pacific regions [Cardosa *et al*., 2003]. Unfortunately, the current treatment only addresses the symptoms [Tan *et al*., 2006]. There is no specific EV71 antiviral available to date [Tan *et al*., 2006]. In light of the pressing EV71 situation faced by the Asia-Pacific region, development for an effective antiviral is crucial.

Since its discovery, the RNAi pathway is currently one of the most researched pathways due to its potential to be used as an antiviral treatment. This pathway occurs in the cytoplasm of almost all eukaryotic cells [Fire *et al*., 1998]. For this reason, RNAi is suitable for usage as an antiviral against EV71 because EV71 replicates in the cytoplasm. RNAi is triggered when the cell encounters a double-stranded RNA molecule, such as that of a viral RNA [Fire *et al*., 1998]. The cell uses this pathway to process the RNA and using the processed RNA to find other RNA molecules that are complementary to it and cleave it [Tomari *et al*., 2004]. If the RNA encountered by the cell belongs to a virus, the cell uses this pathway to look for similar RNA molecules that has complementary sequences and cleaves it [Tomari *et al*., 2004]. By cleaving the RNA molecules, the virus will not be able to replicate successfully and the cell is protected from infection.

One of the objectives of this study is to use siRNA and RNAi against EV71 replication *in vitro*. The virus strain used was the fatal strain, 5865/SIN/00009 (Accession number AF316321; genogroup B4; designated as Strain 41), that was isolated from patients during the HFMD outbreak in October 2000 in Singapore. A specific region of the EV71 genome was targeted for silencing through RNAi. The region targeted was the 3D polymerase region. That particular region of the genome was chosen because it is not prone to mutation.

The siRNAs used in this project were of three different lengths; 19, 27 and 29 nucleotides long. This leads to the second objective of the study, which is to compare the effectiveness of different lengths of siRNAs. According to the theory of RNAi, the 19mer siRNA will not undergo processing by the Dicer molecule because 19mer siRNA is considered as short double-stranded RNA. Thus, the 19mer siRNA will bypass the first processing step and enter directly into the second step, which is being mounted on to the RISC complex. The 27mer siRNA and 29mer siRNA will undergo the entire RNAi pathway without bypassing any step. The three siRNAs were transfected at different concentrations to show that gene silencing with siRNA is concentration dependent.

To effectively fulfill the two objectives, western blot and plaque assay were carried out. The results were compared to determine the efficacy of the three siRNAs at different concentrations.

2. Background

2.1 Enteroviruses

The Picornavirus family is one of the smallest viruses in the world in terms of size and yet has great genetic complexities [Melnick, 2004]. Its size is about 28nm and has a genome size of approximately 7400 nucleotides [Schnurr, 1999]. Picornaviruses are generally classified to six genera; enteroviruses, rhinoviruses, hepatoviruses, parechovirus, foot-and-mouth disease viruses and cardioviruses [Melnick, 2004]. In the enterovirus genus, it is classified to two main groups; human enterovirus and non-human enterovirus [Melnick, 2004]. Non-human enteroviruses infect a wide variety of animals including bovine, rodent and swine [Melnick, 2004]. Human enteroviruses are sub-classified to echovirus, poliovirus, coxsackievirus groups A and B and other enteroviruses. However as of 1969, all new enteroviruses are numbered instead of sub-classifying them to echovirus, poliovirus and such [Melnick, 2004]. Under the sub-class other enteroviruses, there are four types numbered 68 to 71. Out of these four types, type 68, 70 and 71 are known to infect humans [Melnick, 2004].

Isolation of human enteroviruses is done by performing cell culture. Cell lines that are commonly used for the isolation of human enteroviruses are Rhabdomyosarcoma (Rd), Buffalo Green Monkey Kidney (BGMK) and Vero [Schnurr, 1999].

Some of the special properties of Picornaviruses are that they are stable at a low pH of 3, with the exception of rhinovirus, and they have an optimal temperature for replication at 37°C [Melnick, 2004]. The human gastrointestinal (GI) tract has a suitable acidic pH and temperature, making it the perfect site of replication and growth for the enteroviruses [Melnick, 2004]. Enterovirus infections begin at the oropharynx or the GI tract [Schnurr, 1999]. They will move on to the lymph nodes and subsequently cause viremia [Schnurr, 1999]. At the viremia stage, the enterovirus now has access to enter any tissue in the body. The target tissues of enteroviruses are the central nervous system (CNS), the epidermis,

the cardiac muscles and the pancreas, where the virus can continue to replicate further [Schnurr, 1999].

2.2 EV71 Clinical Manifestations

EV71 is a human enterovirus known to cause HFMD, which can lead to neurological complications [Chong *et al*., 2005]. The disease which was first isolated in the stools of an infant in 1969 in California [Shindarov *et al*., 1979], mainly affects young children under the age of 5. Patients experience painful exanthema and rashes on their hands, feet and limbs, multiple ulcers in their mouths (Figures 1-3), particularly the soft palate, gums and tongue [Melnick, 2004; Singh, 2002] and also fever [Alexander *et al*., 1994]. Life-threatening symptoms include pulmonary edema [Melnick, 2004; Sarnow, 2003] and pulmonary hemorrhage [Melnick, 2004]. If the symptoms are left untreated, the patients could develop severe neurological complications such as encephalitis, aseptic meningitis and acute flaccid paralysis [Chong *et al*., 2005]. In major outbreaks in Malaysia and Taiwan during 1997 and 1998 respectively, patients who had died from HFMD suffered from pulmonary edema and pulmonary hemorrhage after the onset of brain-stem encephalitis [Hellen, 1995].

However, EV71 is not the only cause of HFMD. Other viruses known to cause HFMD are Coxsackievirus A16 (CA16), Coxsackievirus A5 (CA5) and Coxsackievirus B3 [Oberste *et al*., 1999]. Out of these other causes of HFMD, the most notable is CA16. Clinically, the herpangina symptoms caused by EV71 and CA16 are virtually indistinguishable but EV71 infection has more association to neurological complications and has much higher fatality rates than CA16 [Kuo *et al*., 2002].

The neurological presentations of EV71 infections reported include muscle spasms, loss of muscle coordination, paralysis and vomiting [Ziegler *et al*., 1995]. During an EV71 outbreak in Taiwan in 1998, a study was conducted to determine the symptoms presented by infected patients. It was found that 87% of the patients were under the age of five and out of the 34 patients which were studied, 30 of them suffered from brain stem

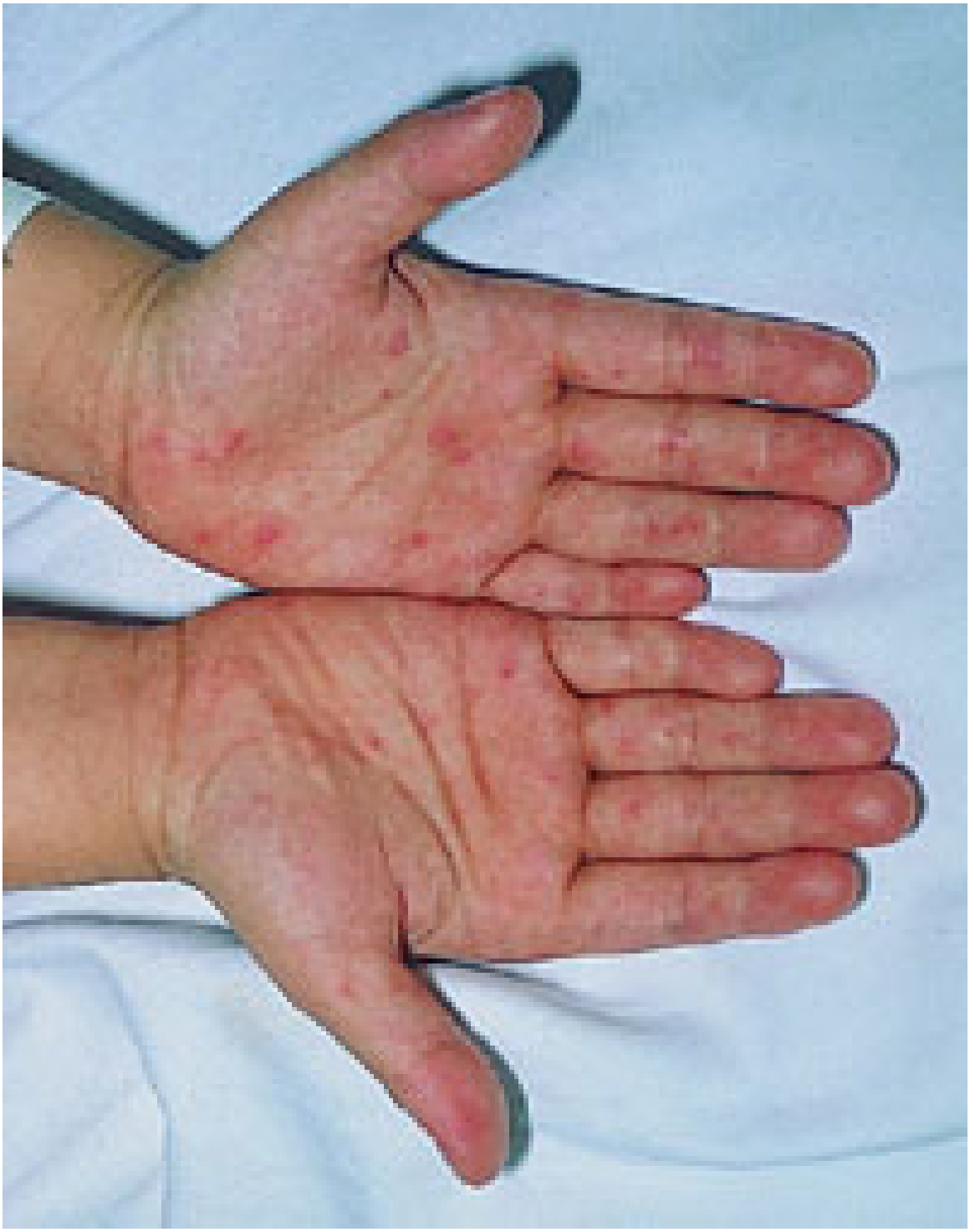


Figure 1: Exanthema on the hands

One of the common clinical manifestations of HMFD is the presence of exanthema and rashes on the palm of the hands.
[http://english.pravda.ru/img/idb/hand21.jpg]

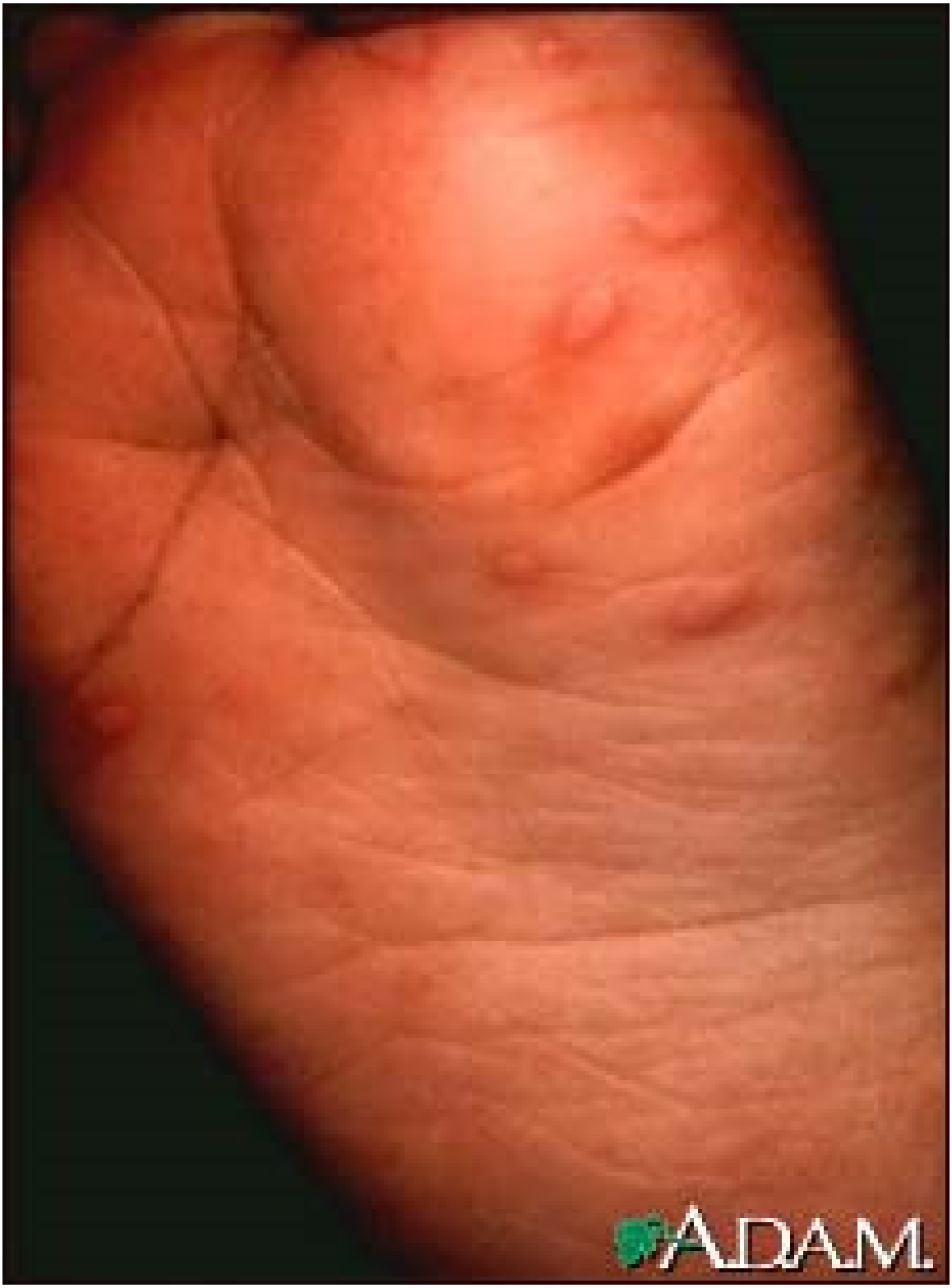


Figure 2: Exanthema on the feet
Blister-like formations on the soles of the feet are characteristic symptoms in the HFMD cases. Fluid from the blister-like formations may be taken for diagnostic purposes. [http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/2312.jpg]

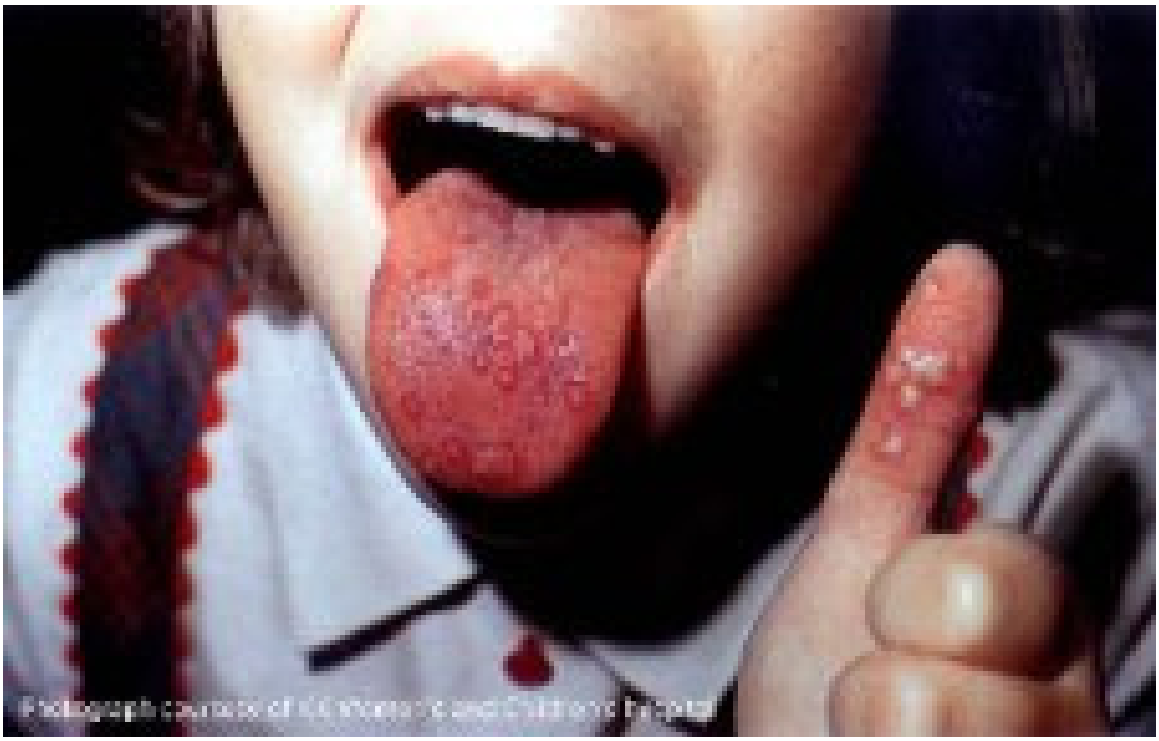


Figure 3: Multiple ulcers on the tongue
Painful ulcers in the oral cavity and blisters on palms are typically seen amongst HFMD patients. [http://www.sgpreschoolzone.com/resources/feature/images_hfmd/mouth.jpg]

encephalitis while the remaining four patients had either aseptic meningitis or meningomyelitis [Ziegler *et al*., 1995]. Magnetic resonance image (MRI) scans revealed that EV71 had infiltrated into the medulla, pons and the midbrain and caused necrosis, suggesting that those were the targeted tissues [Ziegler *et al*., 1995]. However, not all of the patients with brain stem encephalitis tested positive in for EV71 in their cerebral spinal fluid (CSF) samples. The most common sample that tested positive for EV71 using cell culture was throat swabs followed by stool samples [Ziegler *et al*., 1995]. This further confirms that viral replication occurs in the alimentary canal.

2.3 EV71 Epidemiology

Since its discovery in California in 1969, there had been many reports of HFMD outbreaks in Bulgaria, Hungary, Malaysia and Taiwan between late 1990s and most of 2000s [Ho *et al*., 1999; Melnick, 2004]. Table 1 shows a summary of the epidemiology of EV71.

2.4 Genomic Structure of EV71

As part of the Picornaviridae family, enterovirus 71 (EV71) is a virus with only one single-stranded positive RNA strand [Chong *et al*., 2005]. It is a non-enveloped virus with a genome size of 7411 nucleotides [Chong *et al*., 2005]. The genomic structure of EV71 is mainly divided into 5 sections; 5' untranslated region (UTR), P1, P2, P3 and 3' UTR (Figure 4). Its single open reading frame (ORF) is situated at the 3' end of the 5' UTR and the 3' end of the P1 region. The P1 region encodes for four different structural proteins; namely, VP1, VP2, VP3 and VP4. The P2 region encodes for three non-structural proteins, 2A, 2B and 2C, while the P3 region encodes for four non-structural proteins, 3A, 3B, 3C and 3D.

The 5' and 3' UTR can be found in all Picornaviruses. While the 3' UTR is basically a poly-A tail variable in length, the 5' UTR is slightly more complicated. It was found in a former research on the 5' UTR of poliovirus, which is also under the Picornavirus family,

Table 1: An Overview of the Epidemiology of Hand-Foot-Mouth Disease

Year	Country	Cases	Deaths
1969-73	California	20	1
1972-73	Australia	49	None
1973	Sweden	195	None
1973	Japan	335	None
1975	Bulgaria	750	44
1977	New York	29	None
1978	Hungary	1550	45
1986	Australia	140	None
1987	Philadelphia	5	None
1987	Hong Kong	5	None
1987	Japan	692	None
1997	Malaysia and Sarawak	2628	41
1998	Taiwan	126109	78
1999	Australia	14	None
2000	Malaysia	1725	None
2000	Singapore	3790	4
2001	Hong Kong	284	None
2005	Vietnam	764	None
2005	Taiwan	60	4
2005	India	>200	None
2005	China Zhejiang	101	None
2006	Brunei	140	1
2006	Sarawak	>13000	13
2006	Singapore	3000	None
2008	Taiwan	311	10
2008	Singapore	21000	1
2008	Vietnam	2000	None
2008	Malaysia	1943	None
2008	China	176321	40

that 5' UTR plays an important role in RNA synthesis and translation [Alexander *et al*., 1994] because it contains the internal ribosomal entry site (IRES), which allows cap and end-independent translation of mRNA [Sarnow, 2003]. Later, it was found that all Picornaviruses contained the IRES in their 5' UTR [Alexander *et al*., 1994].

The EV71 genome is divided into three sections; P1, P2 and P3, which encode for the structural and functional proteins of the virus. Firstly, the P1 region encodes for four of the viral capsid proteins; VP4, VP2, VP3 and VP1. From X-ray diffractions analysis and electron microscopy, the structure of EV71 was revealed to have icosahedral symmetry [Hellen, 1995] (Figure 5). Each of the surface lattices is made up of 60 units of capsid proteins [Hellen, 1995] and each of the capsid protein is made up from the four proteins encoded by P1. The layout of each capsid is such that the VP4 protein is kept inside the structure formed by VP1, VP2 and VP3 [Hellen, 1995]. Over the years, VP1 has shown to be of great interest, especially in the development and relation to the immune system. Through comparisons with other Picornaviruses, VP1 is shown to be the most immunodominant of the four structural proteins [Oberste *et al*., 1999], meaning that it is the most easily-recognized by the immune system.

The next region from the 5' end of the P1 region is the P2 region. This region encodes for three proteins; 2A, 2B and 2C. These proteins are involved in the replication of the EV71 virus. The functions of the 2A protein are to cleave the P3 proteins, specifically the 3C and 3D polypeptides [Kuo *et al*., 2002], and to initiate the IRES translation system to commence viral translation [Ziegler *et al*., 1995]. It was also found that Enteroviruses undergo recombination frequently and the 2A region is a hotspot for recombination [Lukashev *et al*., 2003]. Not much is known about the 2B protein, but recent studies suggested that the Picornvirus 2B protein is a small hydrophobic protein that situates itself in the endoplasmic reticulum (ER) and the Golgi apparatus [de Jong *et al*., 2008]. The study showed that enterovirus 2B protein is able to bring down the calcium concentrations in the two organelles by forming transmembrane pores and also inhibit

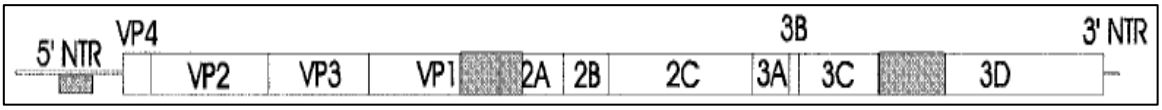


Figure 4: Genomic structure of EV71

The ORF is flanked by 5'UTR and 3'UTR. The P1 region consists of VP4, VP2, VP3 and VP1, the P2 region consists of 2A, 2B and 2C and the P3 region includes 3A, 3B, 3C and 3D. [Lukavesh A. N., 2003]

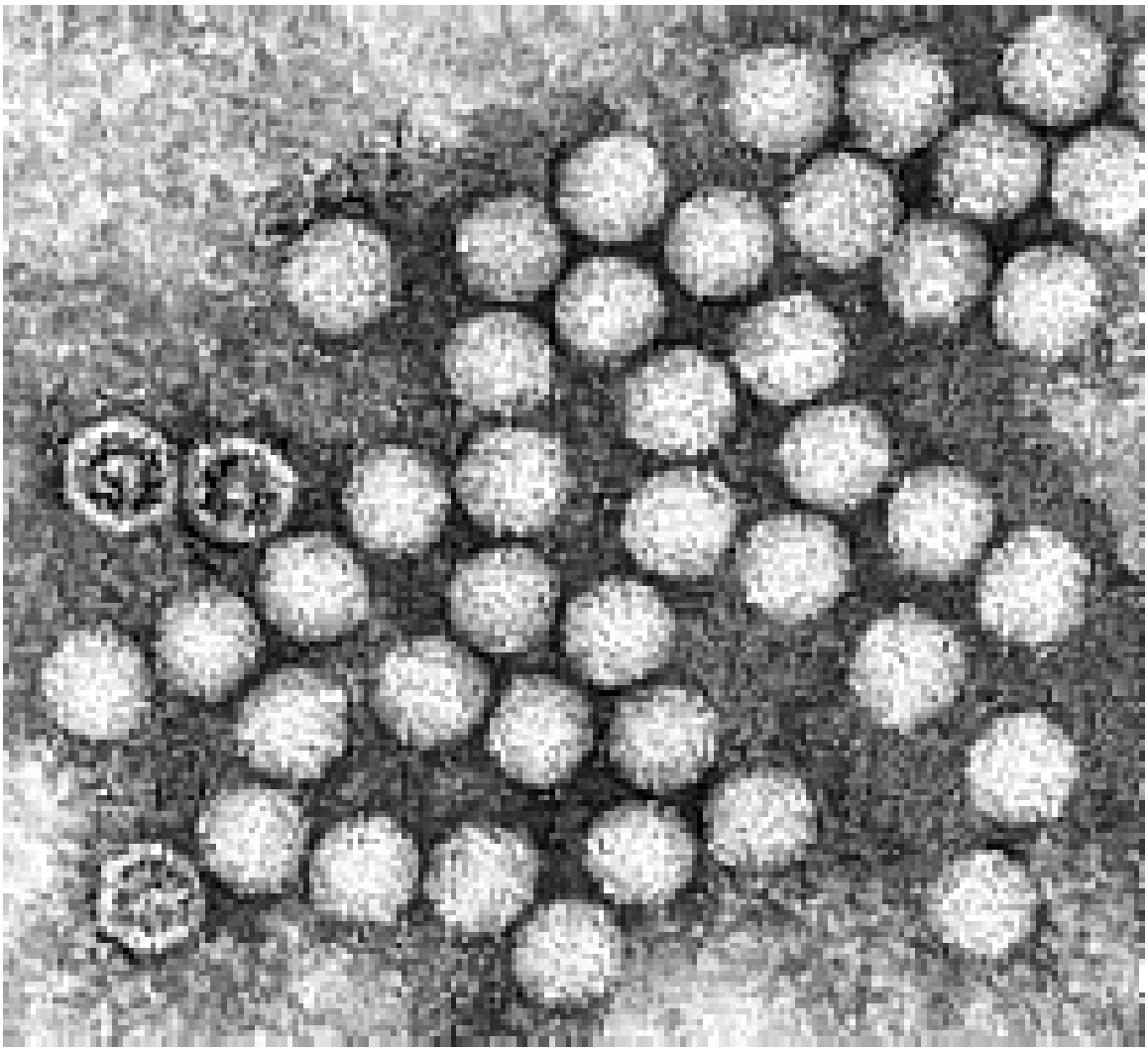


Figure 5: Electron microscopy of EV71
Transmission electron microscope image shows the isocahedral shape of EV71. Each surface is made of 60 capsid proteins.
[http://www.clinical-virology.org/gallery/images/em/enterovirus.gif]

protein trafficking [de Jong *et al*., 2008]. The 2C protein is the most highly conserved viral protein of all the Picornavirus proteins and was found to be closely related with the replication complex-associated vesicle, suggesting involvement of the 2C protein with the replication process [Tang *et al*., 2006].

The P3 region encodes for four polypeptides; 3A, 3B, 3C and 3D. The main polypeptides in this region are 3C and 3D. The 3C protein is a protease that cleaves the P1 polypeptide to yield the four individual structural proteins [Sean P, 2008] and 3D encodes for the RNA-dependent RNA polymerase which EV71 uses for its replication [Sean P, 2008].

2.5 Diagnosis of EV71

HFMD is transmitted from human to human through direct contact with EV71, CA16 or any other enteroviruses known to cause HFMD. From a HFMD patient, the viruses can be found on the nose, mouth, hands, feet, stool, saliva and the fluid in exanthema [http://www.cdc.gov]. The standard diagnosis of EV71 is through cell culture followed by neutralization test based on the Lim Benyesh-Melnick (LBM) pool [Lim *et al*., 1960]. During the 1950s, Lim *et al.* developed a serotypic identification method using the serotype-specific antibodies found in animal serum to neutralize the enteroviruses that they had cultured [Lim *et al*., 1960]. If the results of the neutralization tests were positive, it would mean that the serum contained the specific antibodies for the enterovirus that it was inoculated with [Lim *et al*., 1960].

However, the cell culture method is a long and arduous process that requires at least a week [Singh *et al*., 2002; Tan *et al*., 2006]. In 1989, research was carried out on a diagnosis method using polymerase chain reaction (PCR) [Rotbart, 1990]. In the study, the enteroviruses such as Coxsackieviruses and Echoviruses were collected from the CSF samples of the patients and viral RNA was extracted [Rotbart, 1990]. The region that was to be amplified was near the 5' end of the RNA sense strand [Rotbart, 1990]. Two primers, 20 to 25 nucleotides, and a probe were designed to bind to the 3' end and 5' end and the central part of the targeted region respectively [Rotbart, 1990]. Being RNA

viruses, reverse transcription HFMD is transmitted from human to human through direct contact with EV71, CA16 or any other viruses known to cause HFMD. On a HFMD patient, the viruses can be found on the nose, mouth, hands, feet, stool, saliva and the fluid in exanthema [http://www.cdc.gov].

Gel electrophoresis was done to identify the result of the PCR run and X-ray film blots were captured to show the estimated concentration of PCR products through the intensity [Rotbart, 1990]. The use of PCR greatly improved the time taken to diagnose patients [Rotbart, 1990]. This method was supported by another research team in 2000, when an EV71-specific assay was developed to rapidly distinguish patients who are infected by EV71 or CA16 [Brown *et al*., 2000]. The assay developed exploited the technology of reverse-transcription PCR (RT-PCR) and the region that was amplified was the VP1 region [Brown *et al*., 2000]. Following 2000, RT-PCR had been widely accepted as a method of diagnosis for EV71, especially during outbreaks [Yan *et al*., 2001; Tsao *et al*., 2002; Tsao *et al*., 2006]. The advantages of RT-PCR are numerous compared to the neutralization method. Not only time for diagnosis can be shortened, samples with low viral titre such as CSF can also be used for detection [Rotbart, 1990].

Another method of diagnosis by immunofluorescence assay (IFA) [Ho *et al*., 1999]. This diagnostic method is used after infecting a monolayer of cultured cells and staining the cells with fluorescent dyes. Cell lines used for viral isolation include Vero, RD cells and MRC-5 cells [Ho *et al*., 1999]. As this method is dependent on cell culture, the main problem faced is similar as that faced by the neutralization method; the long time it takes to ‘rule in’ the EV71 infection.

In 2002, detection of IgM in the sera of EV71 patients by enzyme-linked immunosorbent assay (ELISA) proved that it could be effective as a diagnosis method [Tano *et al*., 2002]. The advantage of using this technique for diagnosis is that it is rapid [Tano *et al*., 2002]. However, the downside is that the IgM is a pentamer made of five identical subunits, making IgM non-specific due to its multiple antigen-binding sites hence, giving rise to high probability of cross-reactions and false positive results.

With many potential unreliability and problems faced in the diagnostics, it would be quite difficult to provide the right treatment for HFMD patients. Therefore, there is an urgent need for accurate and proper diagnostic method

2.6 Treatment for EV71 Infection

There are currently no effective vaccines or antiviral therapies against EV71 [Tan *et al*., 2006]. Managing and keeping the infection under control can be difficult but it is an important aspect that may mean life or death for the patient.

As with viral infections, cell-mediated immunity is initiated in an attempt to fight off the enterovirus infections [Schnurr, 1999]. The B lymphocytes will produce an antibody specific to the type of enterovirus, making the immunity type-specific and long-lasting [Schnurr, 1999]. Pulmonary edema and pulmonary hemorrhage are the most common causes of death for EV71 patients. In a study on immunological responses during EV71 infection, the fatality rate for pulmonary edema was 64% [Wang *et al*., 2003]. Autopsy results from patients who had died from pulmonary edema revealed that cardiac function and pulmonary pressure were all normal [Wang *et al*., 2003]. However it was reported that the concentration of cytokines was elevated in those patients, especially interleukin-10 (IL-10), IL-6, IL-13, interferon gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) [Wang *et al*., 2003]. The white cell count was decreased, particularly for CD4+, CD8+ T cells and natural killer (NK) cells [Wang *et al*., 2003]. This could be caused by increase in leukocytosis and thrombocytosis [Wang *et al*., 2003]. Hence, it was suggestive that the pulmonary edema was caused by excessive inflammation in the periphery and CNS [Wang *et al*., 2003].

A group of compounds called ‘WIN’ group was shown to be have the most potential as an antiviral drug against EV71. The antiviral activity of ‘WIN’ group compounds is the prevention of virus uncoating after the virus has been bound to the receptor [Peaver *et al*., 1999]. Pleconaril (VP-63843) is one of the drugs in the ‘WIN’ group which has shown

significant effects against enteroviral meningitis [McMinn, 2002]. Initially used against the common cold caused by rhinoviruses, which is also in the Picornavirus family, pleconaril is now undergoing Phase III of clinical trials in the United States [Romero, 2001]. The clinical studies had shown that the duration and intensity of enteroviral meningitis symptoms were reduced [Romero, 2001]. However, pleconaril was also shown to have limited effectiveness against EV71 [Peaver *et al*., 1999].

One of the treatments used in the outbreak in Taiwan in 1998 was intravenous immunoglobulins (IVIG) [Wang *et al*., 2006]. IVIG is used to help patients with low gammaglobulins (hypogammaglobulinaemia) to strengthen their immune responses against infections [Chun *et al*., 2008]. In the case of EV71, it was administered to boost the immune responses against the EV71 infection. Brain stem encephalitis is one of the severe symptoms of EV71 infection which arises from the increase in cytokines mentioned above. Although IVIG was not reported to have been effective in the treatment of EV71 infection, a study conducted in 2006 showed that IVIG lowered the pro-inflammatory cytokines IL-4, IL-6 and IL-8 in patients with brain stem encephalitis and pulmonary oedema to the point where they were undetectable with ELISA [Wang *et al*., 2006].

Recently, another study showed that *Salvia miltiorrhiza* (Danshen) had the ability to inhibit EV71 replication in vitro. This drug is found in Chinese herbs and was tested in Vero, RD and MRC-5 cells. All the cell lines were shown to have reduced cytopathic effects, suggesting that *S. miltiorrhiza* has the potential to be used as an antiviral agent against EV71. (Wu *et al*., 2007)

A more recent study involving Chinese herbal medicine was found to induce interferons and antiviral activities against Japanese encephalitis virus (JEV) and EV71 [Lin *et al*., 2008]. The active component in this herb is aloe-emodin which significantly activated interferon-stimulated response element and gamma-activated sequence [Lin *et al*., 2008]. IFN responses are induced naturally in the body during a viral infection. They assist the immune response by activating the NK cells and macrophages. The research group

conducted dose-dependent and time-dependent studies using aloe-emodin against JEV and EV71 in cultured cells [Lin *et al*., 2008]. The replication of these two viruses was inhibited via the interferon signalling responses [Lin *et al*., 2008]. The cultured cells showed low cytotoxicity to aloe-emodin and significantly up-regulated IFN-stimulated gene expression such as double-stranded RNA protein kinase [Lin *et al*., 2008]. There was also increased activation of nitric oxide which activates guanylate cyclase, an enzyme that catalyzes the formation of cyclic GMP. Cyclic GMP is known to activate the protein kinases.

2.7 RNA Interference

The RNAi pathway, which was discovered by the team of Andrew Fire and Craig Mello, is involved in gene silencing [Fire *et al*., 1998]. RNAi is triggered when a cell comes across a double-stranded RNA (Figure 6). The double-stranded RNA, which may be a viral RNA or mRNA, is first encountered by Dicer, a protease that processes the long double-stranded RNA to small interfering RNA (siRNA) which is about 19 to 23 nucleotides in length with two-base 3' overhangs [Nykanen *et al*., 2001]. The siRNA is then mounted onto RNA-induced silencing complex (RISC) by the RISC loading complex (RLC) [Liu *et al*., 2003]. RISC constitutes of three main domains; Piwi, Argonaute (Ago2) and Zwille (PAZ) while the RLC has two domains: DCR2 and R2D2 [Tomari *et al*., 2004]. R2D2 is a domain which binds to the more thermodynamically-stable end of the siRNA, leaving the less thermodynamically-stable end of the siRNA to bind to DCR2 [Tomari *et al*., 2004]. In this manner, R2D2 is responsible for the orientation of the siRNA in RISC. The Ago2 domain in RISC binds to the siRNA on the RLC, thereby removing the siRNA from the RLC domains and transferring it to RISC [Hammond *et al*., 2001; Okamura *et al*., 2004; Rand *et al*., 2004]. The transfer of siRNA from RLC to RISC is facilitated by Armitage, a DEAD-box helicase [Tomari *et al*., 2004]. The siRNA is then unwound to its sense and anti-sense strands and the sense strand will be degraded. The remaining anti-sense strand will act as a guide, leading RISC to the targeted mRNA [Rand *et al*., 2005; Matranga *et al*., 2005]. The RISC complex will expose two to eight nucleotides on its surface as recognition to viral mRNA [Tomari *et*

al., 2004]. Once located, the siRNA will form an A-type helicase with the mRNA, which positions the mRNA to the Ago2 Piwi endonuclease domain for cleavage [Tomari *et al*., 2004]. Ago2 cleaves the phosphodiester of the complementary mRNA with Mg2+ as a catalyst [Schwarz *et al*., 2004; Martinez *et al*., 2004]. The viral mRNA is now destroyed and viral replication inhibited. The RISC complex releases the mRNA and is recycled by going through another round of mRNA cleavage [Hutvagner *et al*., 2002].

In many ways, the RNAi pathway is seen as a potential antiviral treatment. However, viruses are known to mutate frequently. If this is so, the pre-mutated siRNA will be unable to guide the RISC complex to the targeted mRNA. If the mutation is in the central region, the binding of the siRNA to the mRNA will not be affected but the cleavage may be interfered [Liu *et al*., 2004].

2.8 siRNA Design

For RNAi to be triggered and optimized, the design of siRNA is of importance as it will affect the effectiveness and potency of gene silencing [Reynolds *et al*., 2004; Table 2]. One of the more important criteria for effective gene silencing using siRNA is that there has to be low G/C content in the siRNA [Reischl *et al*., 2008]. In a study of siRNAs, highly functional siRNA had G/C content of 36% to 52% [Reischl *et al*., 2008]. Having a siRNA with G/C content lower than 30% would affect the recognition of siRNA to mRNA while G/C content that is more than 52% would affect the unwinding process [Reynolds *et al*., 2004]. Administering this criterion alone saw a 1.2% to 3.2% increase in functionality [Reynolds *et al*., 2004].

The second requirement is to have low internal stability in the 5' anti-sense region. This means that it would be ideal to have A/U saturation in the 5' anti-sense region [Reischl *et al*., 2008]. It was proven that having low internal stability at this particular region is required for effective silencing, duplex unwinding and entry of anti-sense into the RISC complex [Reischl *et al*., 2008]. A/U saturation also creates low thermodynamics. In the RNAi mechanism, the R2D2 domain will bind to the thermodynamically-stable end of

the siRNA while the less thermodynamically-stable end will be left to the DCR2 domain. There is a bias toward the adenosine at the 5' region, making it the less thermodynamically-stable end [Dykxhoorn *et al*., 2006]. This is the end that DCR2 will bind to. The 3' end tends to have slightly higher saturation of G/C which makes it thermodynamically-stable, allowing R2D2 to bind to it [Dykxhoorn *et al*., 2006].

The third requirement is to avoid having repeated internal sequences in the siRNA structure. Having many repeated internal sequences will bring about hairpin-like formations within the siRNA instead of a linear structure [Reischl *et al*., 2008]. Overall, the three requisites described above are used to stabilize the structure and positioning of the siRNA in RISC [Reischl *et al*., 2008].

It was found in a research conducted by Reynolds *et al*. that siRNA efficiency increased by 30.6% when their siRNA had a uridine in the tenth position [Reynolds *et al*., 2004; Reischl *et al*. 2008]. The reason behind this is that although Ago2 could cleave anyplace in the target mRNA, there was a bias toward cleavage at the tenth position with the uridine [Reynolds *et al*., 2004]. Further research was carried out in search of bias and prejudices on the type of nucleotide present at a particular position on the siRNA, and more of such cases were uncovered. Adenosine at position 3 of the sense strand increased the efficiency of Ago2 cleavage of mRNA while guanine at position 13 of the sense strand saw prejudice against the effectiveness of RISC on the target mRNA [Reynolds *et al*., 2004]. The rationality behind this is still unknown but it was speculated these bias and prejudices affected the binding of RISC to mRNA or the recycling of the RISC complex [Reynolds *et al*., 2004].

One reason that siRNA is used to trigger RNAi instead of using double-stranded RNA is because double-stranded RNA is known to activate Toll-like receptor (TLR) 3 [Hornung *et al*., 2005]. Under *in vivo* circumstances, viral replication in a normal individual will trigger TLR3. During viral replication, the virus synthesizes long double-stranded RNA which will be later translated to proteins. These double-stranded RNA will activate TLR3, which has evolved to detect pathogen-specific molecules [Hornung *et al*., 2005]. TLR3

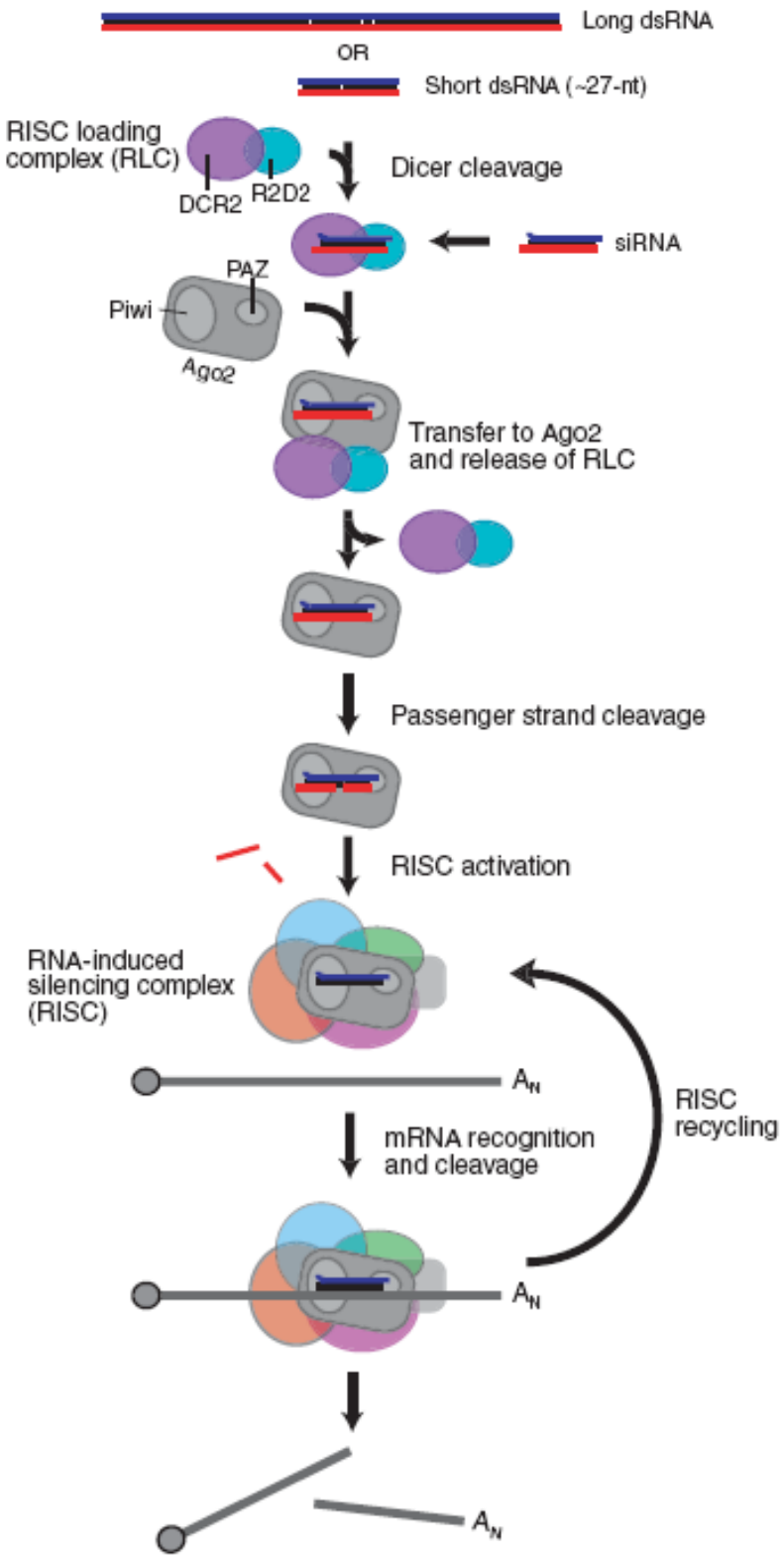


Figure 6: RNAi pathway
[Dykxhoorn, D. M., 2006]

will in turn trigger the IFN pathway by activating immune response [Hornung *et al*., 2005]. The activation of IFN is effective in antiviral activity but this does not prove the effectiveness of RNAi. In a study done by Hornung *et al*., siRNAs of different sequences were transfected in plasmacytoid dendritic cells (PDC) to confirm their hypothesis that the sequence of siRNA will affect the triggering of the IFN pathway [Hornung *et al*., 2005]. PDC were used because they are immune cells that contained plenty of TLR [Hornung *et al*., 2005]. At the end of the study, not only was the hypothesis was confirmed, but they also managed to uncover several sequences that trigger the IFN pathway. It was found that the sense strand of the siRNA was responsible for the activation of the IFN pathway; the anti-sense strand was not involved [Hornung *et al*., 2005]. One of the sequences that activated the IFN pathway was 5' → 3' GUCCUUCAA [Hornung *et al*., 2005].

The sixth criterion is basically avoiding any sequences that have homology to unintended targets [Reynolds *et al*., 2004]. A BLASTn search should be conducted before deciding the sequence of siRNA [Reynolds *et al*., 2004]. The siRNA could trigger the RNAi pathway against another gene that might be essential for the cell's growth [Reynolds *et al*., 2004].

Table 2: List of criteria for design of efficient siRNA

Criteria	Rationale	References
G/C content is to be kept between 36% and 52%	G/C content less than 30% will affect the interaction of siRNA-loaded RISC to target mRNA. GC content more than 56% will affect the unwinding of siRNA.	Reischl *et al*., 2008
Low internal stability at the 5' anti-sense region created by A/U saturation	Low internal stability is equivalent to low thermodynamically-stability. The DCR2 domain of the RLC will bind to the less thermodynamic end of the siRNA while the R2D2 domain of the RLC will bind to the more thermodynamic end of the siRNA. Thus, this criterion will affect the orientation of the siRNA in the RLC and RISC.	Reischl *et al*., 2008
Avoid repeated sequences within the siRNA	Having repeated sequences within the siRNA structure will increase the chances of hairpin-loop-like formations.	Reischl *et al*., 2008
Nucleotide bias to uridine at the 10^{th} position, to adenosine at the 3^{rd} position and against guanine at the 19^{th} position	Ago2 tends to cleave the mRNA at the 10^{th} position where uridine is positioned. The reasons bias toward adenosine and prejudice against guanine are still unknown but it was speculated that it has to do with the binding of RISC to the target mRNA or the recycling of the siRNA-loaded RISC.	Reynolds *et al*., 2004 Reischl *et al*. 2008

Avoid sequences that triggers the IFN pathway such as 5' → 3' GUCCUUCAA	Such sequences trigger the immune system through activation of type-1 IFN and the release of IFN-α and IFN-β. The activation of IFN responses indicates that the RNAi pathway was not triggered.	Hornung *et al*., 2005
Avoid sequences that have homology to unintended targets	BLASTn search should be conducted to avoid unintentional gene silencing of the wrong target.	Reynolds *et al*., 2004

3. Materials and Methods

3.1 Methodology

The objectives of the project are to determine whether siRNAs targeted at the 3D polymerase region of the EV71 genome could be used as a potential antiviral therapy against EV71 and to compare the effectiveness of different lengths of siRNAs. To tackle these two objectives, a plan was devised. Firstly, the sequences of the three siRNAs were designed to target the 3D polymerase region of the EV71 genome. Next, RD cells were cultured for testing. Upon confluency, the cells were seeded into tissue culture plates for the purposes of either plaque assays or western blot (Figure 7).

3.2 Maintenance of RD Cells

The cells used for this study is the Rhabdomyosarcoma (RD) cell line, because they are susceptible to EV71 and EV71 can proliferate well in RD cells. The cells were given generously by Dr Tan Eng Lee from National University of Singapore for the purpose of this research. RD cells were maintained in minimum essential medium (MEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Appendix I). The cells were cultured in T25 tissue culture flasks and maintained at 37°C and 5% CO_2. When the cells were confluent, they were washed with 1X Hank's Balanced Salt Solution (HBSS) (Gibco, USA) or 1X phosphate buffer saline (PBS) to rinse off dead cells, before adding 1X trypsin for trypsinisation. After the cells have detached from the inner surface of the flask, media was added to neutralise the effect of the trypsin and the cells were passaged to new T25 flasks at 1:2 ratio or 1:4 ratio.

3.3 Cryo-preservation of RD Cells

RD cells were preserved in liquid nitrogen to maintain a supply of cells and also to keep the cells in use at a low passage number. Two confluent flasks were required to be frozen

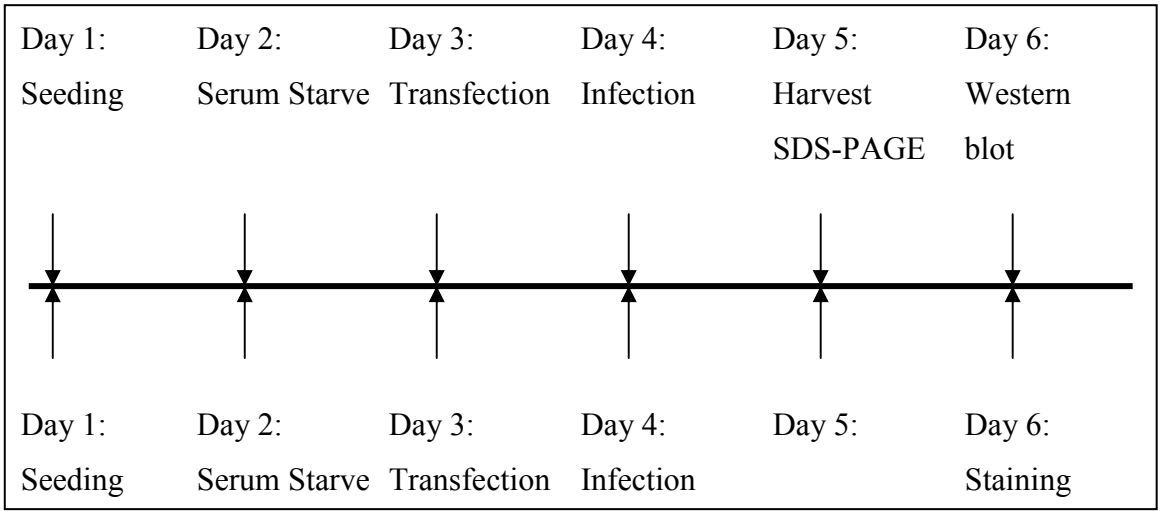


Figure 7: Timeline of methodology
Top timeline shows for western blot protocol. Bottom timeline shows for cell viability plaque assay. For both protocols, cells may be transfected up to 48 hours and infection may be delayed for one day.

to one cryovial at any one time. The media from the flasks were aspirated and the cells were washed with 1X HBSS or 1X PBS to remove dead and suspending cells. The cells were then trypsinised with 1mL of 1X trypsin in each flask. When the cells were completely detached from the flask, media was added to neutralise the trypsin and the cell suspension from both flasks was transferred to a 15mL Falcon tube to be centrifuged at 1000 revolutions per minute (rpm) for 10 minutes at 4°C. Meanwhile, 4.5mL of media and 0.5mL of dimethyl sulphoxide (DMSO) were used to prepare 10% freezing media. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 2mL of freezing media. The cell suspension was transferred into a cryovial and placed in a freezing container (Nalgene®, USA) at -80°C overnight to ensure gradual freezing of the cells before it is transferred to a liquid nitrogen tank.

When cells were needed, the cryovial was retrieved from the liquid nitrogen tank and was thawed immediately at a 37°C water bath. The thawed cell suspension was then transferred to a new T25 flask containing 5mL of media to dilute the toxicity of DMSO in the freezing media.

3.4 Design of siRNA

For the study, three lengths of siRNAs were used; 19 nucleotides, 27 nucleotides and 29 nucleotides. The sequence of the 19 nucleotides used was the same sequence as that of the 19mer siRNA in a previous study conducted by Tan E.L. *et al* (2007). The 27mer 29mer siRNAs were synthesized using the 19mer siRNA sequence as a guide. Since the 19mer siRNA was complementary to a target area in the 3D region of the EV71 genome (Appendix II), eight nucleotides downstream of the 19mer target sequence was added to form the 27mer siRNA. The 29mer siRNA sequence was established in a similar way by adding nine nucleotides upstream and one nucleotide downstream of the 19mer siRNA sequence. To follow the criteria to produce efficient siRNA, the 3' ends of both strands of all three siRNAs were added with UU overhangs. A BLAST search was conducted on the determined sequences of the three siRNAs to ensure that the siRNAs only targeted against the EV71 virus and nothing else. A scrambled sequence of both the 19mer and

29mer siRNAs (19mer Scr and 29mer Scr) with the same base compositions were also synthesized and used as negative controls. All the chemically-synthesized siRNAs were manufactured by Sigma-Proligo, USA (Table 3).

3.5 Seeding of RD Cells

Upon confluence of the cells in the flasks, the RD cells were washed, trypsinised and seeded into each well of a 24-well plate or 6-well plate (Nunc, Germany) at a concentration of $1X10^5$cells/mL. The plate was then incubated at 37°C and 5% CO_2 for growth before using for study.

3.6 Plaque Assay

The plaque assay was conducted to determine the concentration of virus to be used for infection of the cells. When the cells seeded in a 24-well plate had reached confluency and were monolayered, media from the wells were aspirated and 100uL of virus serial diluted to different concentrations of 10 times, 100 times, 1000 times and 10,000 times were pipetted into each well. Each dilution was repeated in quintuplets (Appendix III). The cells were incubated at room temperature with the virus for 1 hour before the virus was aspirated and 500uL of carboxymethylcellulose (CMC) agar (Appendix III) in 2X growth media was placed into each well. The plate was incubated for 48 hours at 37°C and 5% CO_2. After the 48 hours incubation, the CMC agar and viral plaques were removed and the cells were fixed with 4% formalin at room temperature. The formalin was washed away after 30 minutes and the cells were stained with 1% crystal violet for 30 minutes at room temperature. Plaques were seen after the staining procedure and were manually counted. To obtain multiplicity of infection (MOI) of 10, there had to be 100 plaques in the well (Table 4).

Table 3: siRNA sequence

siRNA	Nucleotide sequence	Nucleotide location
19mer	5' – GAAAUUGGCUCGAAUUGUU UU* – 3' 3' – UU CUUUAACCGAGCUUAACAA – 5'	7316 – 7324
27mer	5' – GAAUCUGAGAAAUUGGCUCGAAUUGUU UU – 3' 3' – UU CUUAGACUCUUUAACCGUGCUUAACAA – 5'	7305 – 7324
29mer	5' – AGAAAUUGGCUCGAAUUGUUUUAAUAUUA UU – 3' 3' – UU UCUUUAACCGAGCUUAACAAAAUUAUAAU – 5'	7315 – 7333
19mer Scr	5' – AUUGAAUCGGGCUGUAAUU UU – 3' 3' – UU UAACUUAGCCCGACAUUAA – 5'	
29mer Scr	5' – AAUAGACUCUGGUGUGAAUAAUUUUAAUU UU – 3' 3' – UU UUAUCUGAGACCACACUUAUUAAAAUUAA– 5'	

* UU are the 3' overhangs

Table 4: Calculations for Plaque assay

Multiplicity of infection (MOI) is the average number of virus particles infecting each cell

Plaque forming unit (pfu) is a measure of number of infectious virus particles

Formula

MOI = pfu / number of cells
Pfu = number of plaques / (dilution factor X volume (mL))

Requirements

Target MOI	10
Number of cells / mL	$1X10^5$
Dilution factor	0.001
Volume (ml)	0.1

Calculations

Pfu = 10 X $1X10^5$ = $1X10^6$	
Number of plaques = $1X10^6$ X (0.001X0.5) = 500	(for 6well-plate)
Number of plaques = 500 / 5 = 100	(for 24well-plate)*

*The area of one well in the 6-well plate is approximately 5 times larger than one well in he 24-well plate.

Therefore the number of plaques to be formed in a 24-well plate to achieve a MOI of 10 is 100.

[Capital Biosciences]

3.7 Cell Viability Plaque Assay

RD cells were seeded in 24-well plates and serum starved when cell confluency reached 80%. Transfection was carried out 24 hours post serum starvation and infection was performed 24 hours or 48 hours post transfection. Virus was aspirated from the wells at the end of the infection incubation and 2X CMC agar was pipetted into each well (Appendix IV). The plates were incubated at 37°C and 5% CO2 for 48 hours before the processes of fixation with 4% formalin and staining with 1% crystal violet were executed.

3.8 Transfection and Infection of RD cells

Following seeding in 6-well plates, the cells were serum starved with Opti-MEM I (Gibco, USA) overnight. After that, the cells were transfected with Lipofectamine™ 2000CD (Invitrogen, USA) diluted with Opti-MEM I and different concentrations (3nM, 5nM and 10nM) of the five siRNAs (19mer, 27mer, 29mer, 19mer Scr and 29mer Scr). The cells were transfected for 24 hours or 48 hours before they were infected with MOI 10 of EV71 for 1 hour (Appendix V). The virus was aspirated and fresh media was supplied to the infected cells in the 6-well plates intended for western blot. The cells were incubated at 37°C and 5% CO_2 for another 24 hours before harvesting.

The media from the wells were aspirated and discarded and each well was filled with 300uL of CelLytic M Cell Lysis Reagent (Sigma, USA). The cells were incubated at room temperature for 15 minutes before the cell lysates were collected in Eppendorf tubes and centrifuged at 12000rcf for 15 minutes. The supernatant was transferred to a new set of Eppendorf tubes and stored at -80°C while the cell pellets were discarded.

3.9 SDS-PAGE and Western blot

Harvested proteins were ready for electrophoresis using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Equal volume of sample and 2X loading buffer were mixed and loaded into Ready Gel 10% Tris-HCl Gels (Bio-Rad

Laboratories, USA). Once the pre-cast gel was completely loaded and the electrophoresis tank was filled with SDS-PAGE buffer, electrophoresis was run at 80 volts for 120 minutes. The bands were transferred to a nitrocellulose membrane using iBlot™ Gel Transfer Device (Invitrogen, USA) and western blot was conducted following the procedures from the WesternBreeze® Chromogenic Western Blot Immunodetection Kit (Invitrogen, USA) with the use of EV71 VP1 monoclonal antibody (Chemicon International, USA) at a dilution ratio of 1:5000, (β-actin – 1:5000 also).

4. Results

4.1 Determination of Viral Titre

Before the rest of the experiments could be conducted, the viral titre to be used for the subsequent infections had to be determined. Plaque assay was carried out after seeding using four different dilutions of virus. The virus was serial diluted to 10 times, 100 times, 1000 times and 10,000 times. There were no plaques formed in the wells infected with 10 times and 100 times dilutions of EV71 as almost all the cells had died from the infection (Figure 8). Plaques could be counted in the wells infected at 1000 times dilution. The average number of plaques in each well was 29 (Table 5).

4.2 Protection of RD Cells from EV71-Induced Cytopathic Effects (CPE)

The three siRNAs; 19mer, 27mer and 29mer, designed to target the 3D polymerase region of EV71 were transfected to RD cells at three concentrations; 3nM, 5nM and 10nM. A BLAST search conducted showed that all three siRNAs had no homology to human genes and were specific to the target region.

The efficacy of the three siRNAs in inhibiting EV71 replication was first evaluated when the RD cells were infected at a MOI of 10, 48 hours post-transfection. Inhibition of EV71-induced CPE was observed with a concentration of 3nM of three siRNAs. This was significantly different from the cells transfected with scrambled sequences. The siRNAs actively inhibited replication up to 72 hours post-infection (hpi). Transfected at 10nM, EV71-induced CPE were better inhibited than siRNAs transfection at 3nM and 5nM (Figure 9).

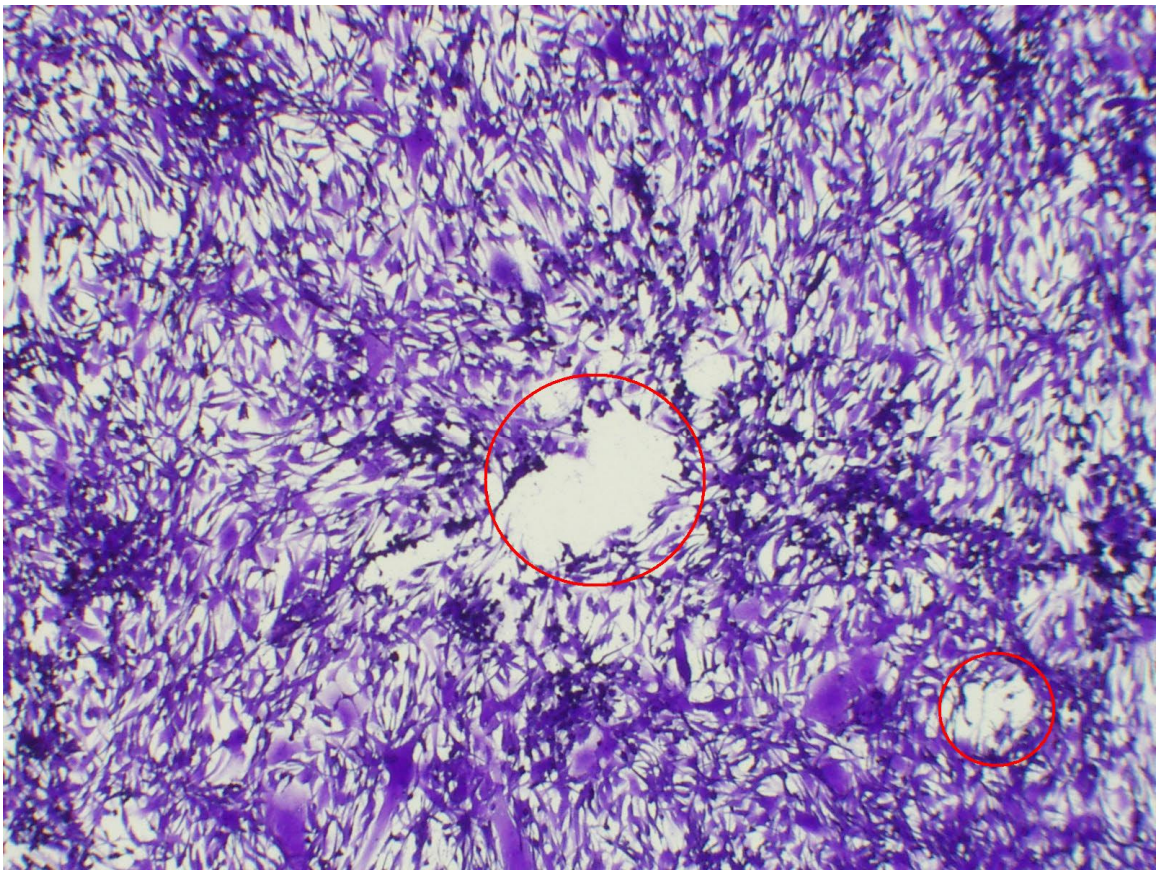


Figure 8: Plaques from plaque assay
The figure shows two plaques indicated by the red circles from the plaque assay. The picture was taken using a microscope camera at 40X magnification.

Table 5: Plaque count in the wells infected with EV71 diluted to 1000 times

Number of plaques	Well 1	Well 2	Well 3	Well 4	Well 5	Average number of plaques per well
Plate 1	29	13	27	11	25	21
Plate 2	36	31	43	40	23	34.6
Plate 3	24	28	38	28	26	28.8
Plate 4	34	36	37	26	33	33.2
Average number of plaques per well, per plate						29.4

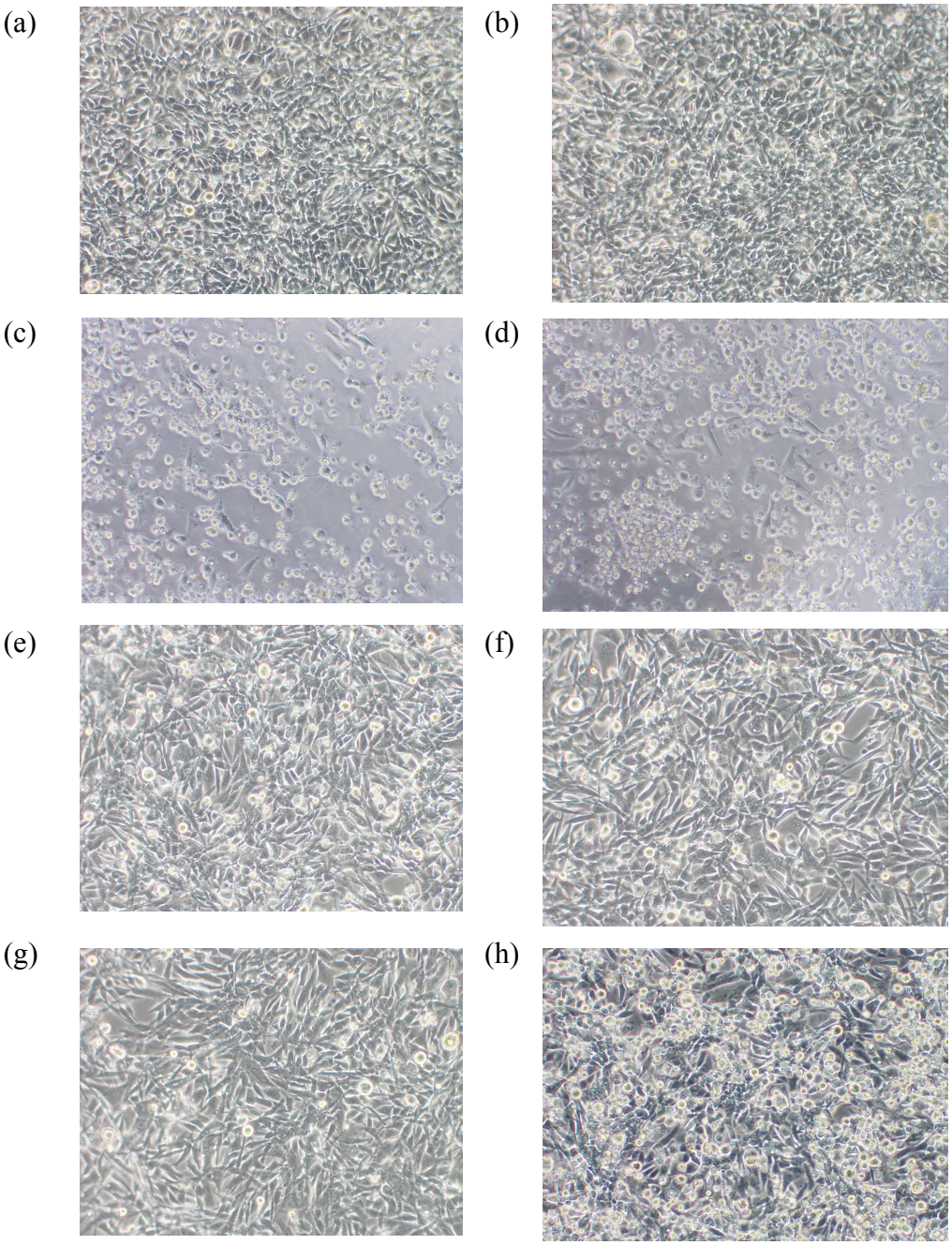


Figure 9: Inhibition of EV71-induced CPE

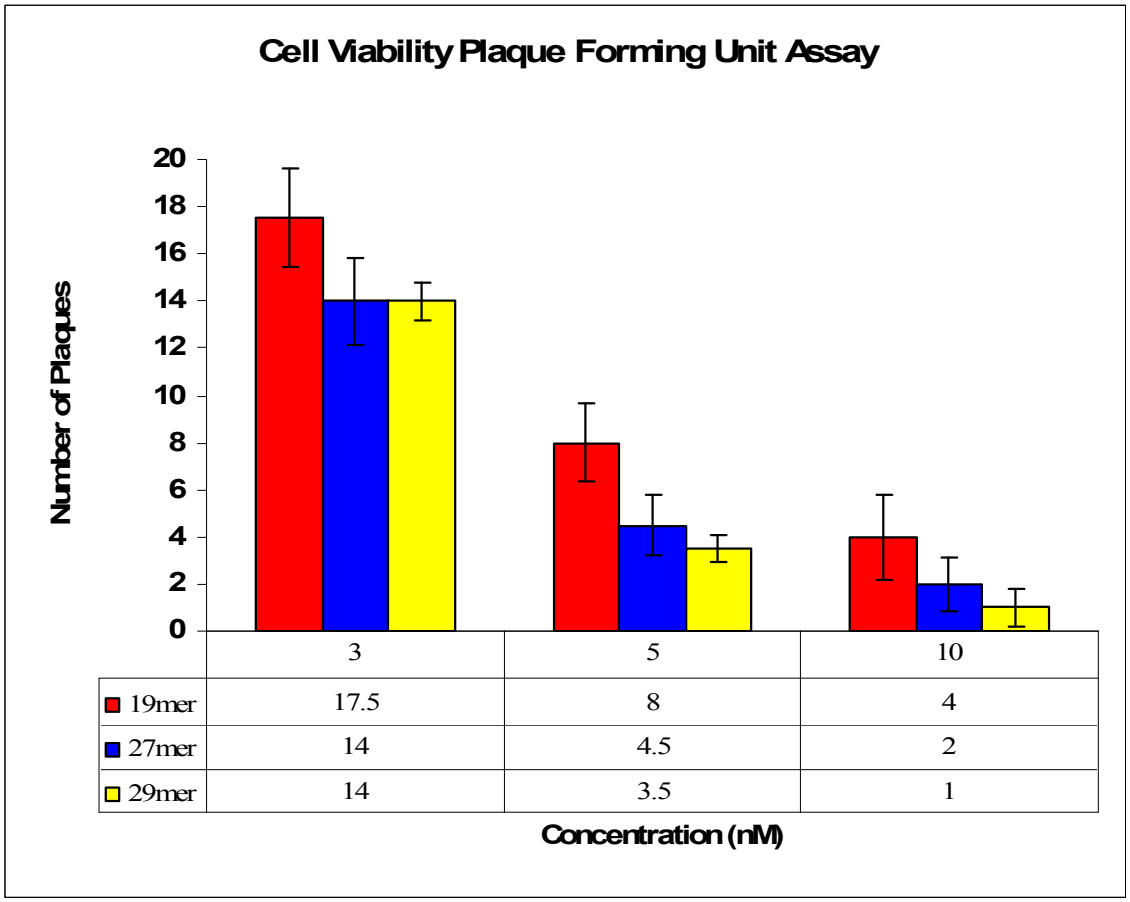
RD cells were transfected with 29mer siRNA at 3nM, 5nM and 10nM before infection at MOI of 10. Morphological changes were observed at 48 hours post-transfection and 24 hours post-infection under a light microscope camera at 40X magnification. (a) untreated and uninfected cells; (b) uninfected cells transfected with 10nM of 29mer; (c) untreated and infected cells; (d) transfection agent treated infected cells; (e) infected cells treated with 3nM of 29mer; (f) infected cells treated with 5nM of 29mer; (g) infected cells treated with 10nM of 29mer; (h) infected cells treated with 10nM of 19mer-Scr. Tests were carried out in two independent tests.

4.3 Inhibition of EV71 Replication by siRNA

Plaque assay was to observe the inhibitory effects of siRNA on EV71. The RD cells were transfected, infected and supplied with CMC agar to allow formation of plaques. The results of the plaque assay showed decreasing trend in the number of plaques with increasing concentration of transfected siRNA (Figure 10).

After the demonstration of EV71-induced CPE inhibition in the plaque assay, the next step was to determine the potency of the three siRNAs by studying the inhibitory effects of the three different concentrations of siRNAs transfected. After transfection of the siRNAs, RD cells were infected over the course of 24 hours before harvesting for western blot or staining for plaque assay. The protein detected with western blot was the VP1 structural protein. The protein bands were observed to be less intense as concentration of siRNA transfected increased. The cells transfected with the scrambled sequences were found to have bands at almost the same intensity as the cells that were infected only.

Once the results of the western blot were established, western blot was conducted again on the same set of samples but blotted for β-actin instead of VP1 protein as a loading control to establish the reliability of the results (Figure 11).



	3	5	10
19mer	17.5	8	4
27mer	14	4.5	2
29mer	14	3.5	1

Figure 10: Cell Viability Plaque Assay

The figure above shows the average number of plaques formed from the cell viability plaque assay. The most number of plaques were formed at 3nM for all three siRNAs and the least number of plaques formed was at 10nM concentration. Comparing among the three siRNAs, 19mer had the most number of plaques while 29mer had slightly fewer plaques than 27mer.

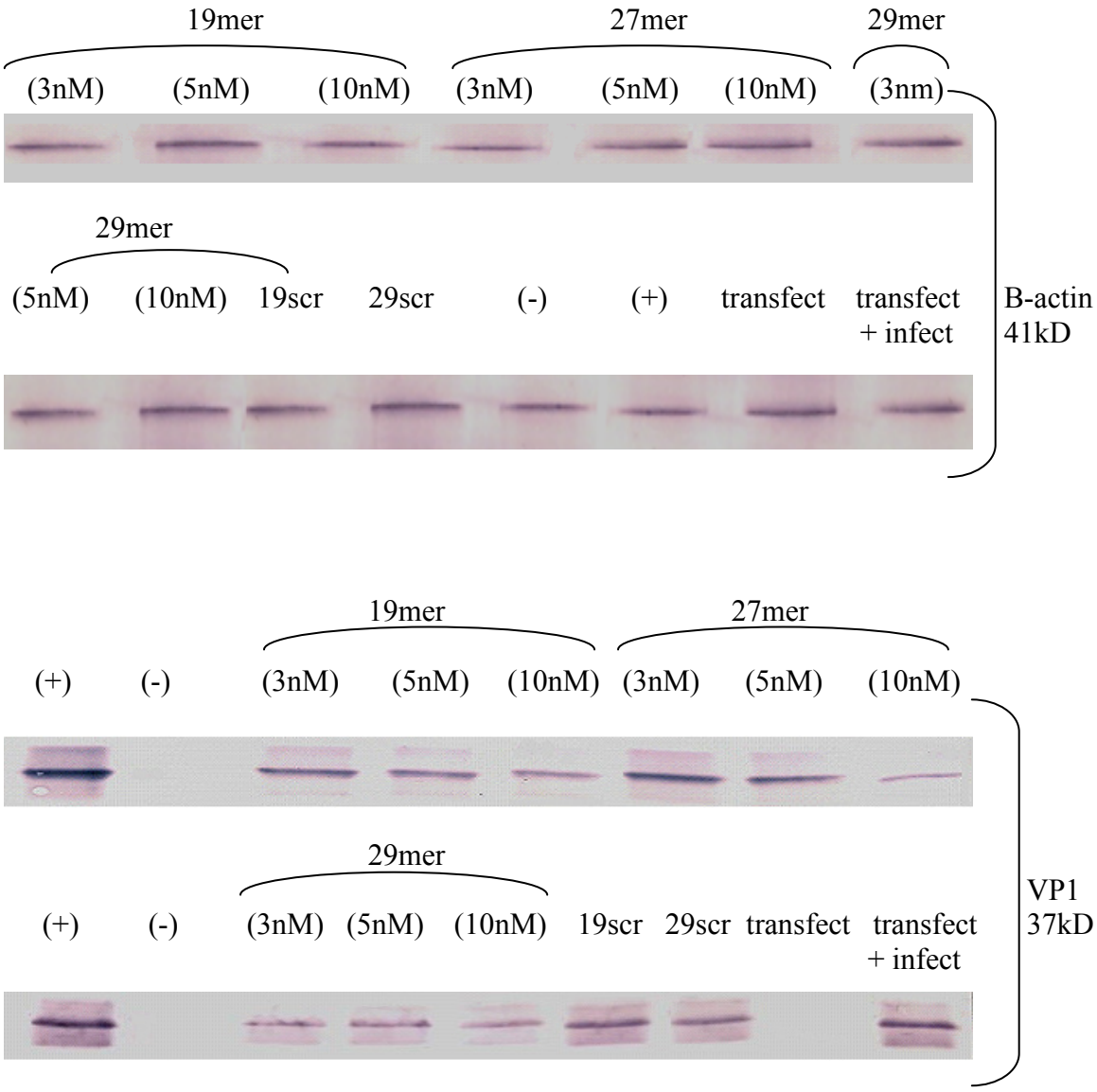


Figure 11: Western blot for VP1 and β-actin
The results of the western blot for VP1 and β-actin are shown above. Both blots for VP1 and β-actin are from the same batch of samples. The band size for VP1 is 37kD and the band size for β-actin is 41kD.

5. Discussion

5.1 RNAi

RNAi has been exploited in many researches against many viruses including human immunodeficiency virus (HIV), Hepatitis B virus and Hepatitis C virus [Huang, 2008]. Some HIV studies have even been proven effective *in vivo* [Kumar *et al*., 2008]. Previous methods explored involved the use of drugs which have variable effectiveness, such as pleconaril, and may also produce undesirable side effects. As RNAi is a naturally-occurring pathway in many eukaryotic cells, using it as a weapon against EV71 is idealistic.

Using chemically-synthesized siRNA provides many advantages over plasmid-derived siRNA because they are more stable than natural double-stranded RNA. They do not degrade easily and can remain effective at room temperature. Another advantage of using chemically-synthesized siRNA is that it is ready-to-use and non-immunogenic [Trulnzch *et al*., 2004]. There is no need for culture of transformed bacteria and harvest of plasmid-derived siRNA, which saves time and reduces the chance of bacterial contamination.

By targeting the 3D region of EV71, there can be no replication as the 3D region encodes for the RNA-dependent polymerase. Removing the polymerase will lead to inhibition of translation of viral proteins or enzymes and eventually inhibit virus replication. In a previous study, it was shown that RD cells transfected with siRNA targeting the 3D polymerase region had the most potent antiviral effect as compared to the 2C, 3C and 3'UTR regions [Chong *et al*, 2005]. However, as the RD cells proliferate, the new cells will not have the siRNA in them and become susceptible to the viral infection. Inevitably, there will be cells that succumb to the virus.

5.2 Plaque Assay

To establish the concentration of virus needed for later parts of the study, plaque assay was conducted. The aim was to achieve a MOI of 10, which was derived to be 100 plaques in a 24-well tray using the formula in Table 6. MOI of 10 for EV71 was shown to be the most suitable viral titre for cells to show almost 100% CPE within two days. In the plaque assay performed, the viruses were serial diluted to four concentrations; 10 times, 100 times, 1000 times and 10,000 times. These four concentrations of virus were infected to RD cells seeded in a 24-well plate. Plaques could be seen in the wells that were infected with EV71 diluted to 1000 times and 10,000 times. The wells infected with EV71 diluted to 10 times and 100 times were infected to the point where there were almost no cells left. With virtually no cells left in the wells, plaques cannot be defined and seen. The number of plaques in the wells infected with virus diluted to 1000 times and 10,000 times were 26 and 5 on average respectively. Seeing as the number of plaques formed by the cells in infected at 1000 times dilution is 26, it would mean that the next best dilution factor that can attain close to 100 plaques is the 0.01. Therefore, the virus concentration used for the later parts of the experiment was 100 times dilution.

5.3 Transfection

Lipofectamine™ 2000 is a transfection agent that is widely used by many researchers due to its ease to use [Invitrogen, USA]. However, this transfection agent can also cause cytotoxicity to cells if used at high concentrations [Invitrogen, USA]. The amount of Lipofectamine™ 2000 used in this study was 1.2uL for 24-well plate and 6.0uL for 6-well plate, which was the suitable volume to contain the siRNA and also at suitable concentration such that the cells would not experience cytotoxicity even after 48 hours (Figure 9b). After transfection with Lipofectamine™ 2000, the morphology of the cells was noted to be rounded but returned to its normal spindle shape after a few hours. This was reported to be a normal phenomenon as the cells were picking up the micelles containing the siRNA [Invitrogen, USA].

Similarly, siRNAs were also reported to be able to induce cytotoxicity to cells [Persengiev *et al*., 2004]. To prove that the RD cells underwent CPE as a result of the EV71 infection and not the siRNA or transfection agent, two controls were set up. One of the wells was treated with transfection agent and a scrambled sequence of siRNA while another was treated with transfection agent only. 48 hours post transfection, the cells did not show signs of CPE; they were still intact and adhered to the bottom of the wells.

Besides RNAi, another pathway can also be triggered by double-stranded RNA. This pathway, called protein kinase R (PKR), is involved in a cascade of pathways, triggering of proteins and enzymes [Garcia *et al*., 2006] and protection against viral infections [Khabar *et al*., 2003]. When it is activated by double-stranded RNA or interferon, PKR will be phosphorylated and its levels are up-regulated. The activated PKR will phosphorylate eIF2α, a cellular protein, which is found downstream of the interferon pathway. Following a cascade of pathways, translation of viral proteins is arrested [Khabar *et al*., 2003]. To ensure that the siRNA transfected did not trigger the PKR pathway but the RNAi pathway, the maximum length of siRNA used was 29 nucleotides. It was found in a previous study that the PKR pathway is triggered when the cell comes across a double-stranded RNA that is more than 30 nucleotides in length [Kim *et al*., 2005]. It was also shown in another study that even at 29 nucleotides the cell would trigger the RNAi pathway instead of the PKR pathway [Kim *et al*., 2005]. Thus, the use of 29mer siRNA still ensures that the RNAi pathway will be triggered.

5.4 RNAi on cell Viability

Plaque assay was performed post transfection to find out if the siRNA could inhibit EV71-induced CPE. As shown by the results, there is a decrease in the number of plaques in the wells as the concentration of siRNA transfected increased. The plaque assay was carried out in three independent tests and the results for all three assays coincided with each other. This means that synthetic siRNAs are capable of inhibiting EV71 replication and that the inhibition was concentration dependent.

The number of plaques also differs among the three siRNAs. The number of plaques formed in the wells transfected with 19mer siRNA was more than those formed in the wells transfected with 27mer and 29mer siRNAs. It shows that 19mer siRNA is not as effective in inhibiting EV71-induced CPE as 27mer and 29mer siRNAs. The Student's T Test performed backs this conclusion (Appendix VI). Table 11b and 11c indicates that there is no significance between 27mer and 29mer at 10nM, which meant that the 27mer and 29mer were equally effective at inhibiting EV71-induced CPE. However, plaque formation is rather subjective to the naked eyes. The plaques formed in the wells are not very clearly defined at macroscopic level and might be misled while counting plaques. Hence, western blot was performed to confirm the results from the cell viability plaque assay.

5.5 Western Blot

The RD cells were infected after they had been transfected for 24 hours or 48 hours. Fresh media was placed in the wells and kept for 24 hours after the cells had been infected for one hour. The cells were then harvested and western blot detecting for VP1 protein was carried out. The results of the western blot proved that inhibition of EV71 replication through RNAi was dependent on the concentration of siRNA. As seen from the western blot result, the intensity of the VP1 band was stronger at 3nM than 5nM and 10nM for all three lengths of siRNAs (Figure 11). However when the intensities of the 10nM bands were compared against the three siRNAs, the sample transfected with 10nM of 19mer siRNA appeared to have the strongest intensity while the sample transfected with 29mer siRNA at an equal concentration appeared to have the weakest intensity. It can be concluded from this that 29mer siRNA is better than 19mer and 27mer siRNAs inhibiting EV71 replication. It was established from previous studies that siRNAs that bypass the Dicer cleavage step would not be as effective at inhibition as those that go through the step [Tan *et al*., 2006]. The Dicer cleavage is the step that starts off the RNAi pathway after it double-stranded RNA triggers the cell [Dykxhoorn *et al*., 2006]. The RLC and RISC will come into the pathway after the double-stranded RNA has been processed by Dicer [Dykxhoorn *et al*., 2006]. Thus, the processing action of Dicer

provides the cue for RLC and RISC to come into play [Dykxhoorn *et al*., 2006]. Since the double-stranded siRNA is cleaved into siRNA of 19 to 23 nucleotides in length, siRNA that were already 19 to 23 nucleotides long will bypass the Dicer cleavage. As the 27mer and 29mer siRNAs underwent the complete RNAi pathway, they were more effective at inhibiting EV71 replication.

To ensure that the trend seen in the western blot results were not due to different loading concentration, western blot was performed again on the same set of samples but blotting for β-actin instead. B-actin is a protein that is found in RD cells and is commonly used as loading control for western blot, immunocytochemistry and immunoprecipitation [http://www.exactantigen.com]. The loading method used for blotting VP1 and β-actin were the same and the results seen on the membrane showed that the intensities of the β-actin bands in all the samples were equal. This indicates that the decreasing intensity of the VP1 band was a result of better inhibitory effects from the increasing concentration of transfected siRNA.

Discussion of the results proved that the inhibitory effects of siRNA were concentration dependent, at least up to 10nM. It was proven in both the cell viability plaque assay and the western blot for VP1. There was an increasing inhibition with increasing concentration of siRNA transfected. The results also indicated that there was a difference in the EV71-induced CPE inhibition among the three siRNAs. 19mer siRNA, though it had significant inhibitory effects, had the weakest inhibitory effects among the three siRNAs. Being quite short, it evaded the first step initiated by the Dicer protease. The full pathway was not encountered by 19mer.

Besides the obvious difference in intensities between 19mer and the other two siRNAs, a less conspicuous difference in intensity between 27.10 and 29.10 was observed. However the difference in intensity was insignificant and thus, it backed-up the results from the Student's T Test for the cell viability plaque assay that the inhibitory effect between 27.10 and 29.10 was insignificant (Appendix VI).

It can then be concluded that EV71-induced CPE could be inhibited by RNAi using chemically-synthesized siRNAs. The inhibitory effect was concentration dependent at least up to 10nM as seen in the decreasing number of plaques in the cell viability plaque forming unit assay and the decreasing intensities in VP1 protein bands in the western blot. The results from both the cell viability plaque assay and the western blot also evidenced that 19mer had the worst EV71-induced CPE inhibition while 27mer and 29mer had insignificant differences in EV71-induced CPE inhibition at 10nM.

5.6 Problems Encountered

Cell culture was an important part of the study. Without the maintenance of the RD cells, the rest of the research could not be carried out. One of the major problems encountered was contamination. There were many problems with bacterial contamination in the cell line and the growth media. Gram staining revealed that the bacteria frequently encountered were Gram-positive rods or Gram-negative rods.

The issue with contamination was resolved by the improvement of cell culture techniques and the addition of antibiotics into the growth media during media preparation. Techniques such as swabbing hands and gloves with 70% alcohol and changing gloves frequently ensured that the cells were kept within a disinfected area. Another measure to keep the cells free from contamination was the use of deionised water instead of regular tap water to humidify the incubator.

During media preparation, antibiotics were added to prevent the growth of bacteria in the growth media. The antibiotics used were a mixture of Penicillin and Streptomycin. In an aliquot of 50mL of growth media, 50uL of antibiotics were added, making up 1% antibiotics. By deploying these techniques, contamination occurred rarely and the rest of the experiment could proceed.

6. Future Developments

Due to the time constraints of this project, there were many other controls that could not be carried out. Given more time and funding, this study could also have been further developed.

6.1 Transfection Efficiency

One of the controls that could have been carried to solidify the transfection efficiency of the transfection agent and the siRNAs is the MTS assay. The MTS assay is based on the principle of changes in colour caused by the enzymatic reactions [University of Chicago, Medical Center]. The chemical MTS is placed into the wells of the cells and an enzyme, mitochondrial reductase, found in the mitochondria of live cells will reduce the MTS to formazan in the presence of another compound called PMS [Cory *et al*., 1991]. Since the enzyme that reduces MTS to formazan is only found in live cells, the intensity of the formazan colour is directly proportional to the number of live cells in the wells [University of Chicago, Medical Center]. The intensity is measured using an ELISA plate reader at 490nm as this assay is typically performed on a 96-well plate [Promega]. The results of the MTS assay will be in numerical format. The larger the number, the higher concentration of formazan is found in the wells, which is proportional to number of live cells in the well. This MTS assay can be done after transfection to verify that the cells did not undergo CPE due to the cytotoxic effects of the transfection agent and the siRNAs.

To ensure that the siRNA was efficiently transfected into the RD cells, another verification assay could have been conducted. By labelling the siRNA to be transfected with fluorescein isothiocyanate (FITC), the transfected RD cells could be viewed under a fluorescence microscope. FITC is a fluorescent compound that is commonly used for the purpose of antibody and probe labelling [Pierce Biotechnology]. Detection of its fluorescence is usually captured by fluorescence microscopes, flow cytometer and immunofluorescence-based assays such as ELISA [Pierce Biotechnology]. It has an

excitation wavelength of about 494nm and an emission wavelength of 518nm [Pierce Biotechnology].

If fluorescence is seen within the cell, it can be assured that the cells were successfully transfected with the labelled siRNA. However, if the fluorescence is seen outside the RD cells, it could be concluded that the siRNAs were not transfected.

6.2 Inhibition Detection Assay

In this study, assays done to prove that siRNA indeed inhibited EV71 replication were western blot and plaque assay. These two protocols showed different view points of the inhibition. The western blot showed inhibition from the viral point-of-view by blotting for the VP1 protein. Decreasing trends of the VP1 protein indicated that EV71 replication was inhibited based on the concentration of siRNA transfected (Figure 9e, f, g). The plaque assay demonstrated viral inhibition from the host cells' point-of view. Formation of plaques is dependent on having live cells in the wells. If most of the cells succumb to EV71 infection, no cells will be left behind and the wells would be empty. Decreasing number of plaques was seen from the plaque assay also meant the increasing number of cells that had not undergone CPE from the virus.

To further bullet-proof the inhibition results, reverse transcription and real-time PCR could have been conducted. Being an RNA virus, the RNA from the virus should first be converted to DNA via reverse transcription before real-time PCR could have been carried out. Real-time PCR was chosen to validate the results because it has the ability to specifically detect for EV71 and could also indicate how much virus was inhibited by studying the Ct values. Ct values represent cycle thresholds, which are the cycle numbers in which the fluorescence is first detected beyond the threshold [Applied Biosystems]. Northern blot is also a possible method of EV71 RNA detection method [University of South Carolina, School of Medicine]. However, northern blot requires a larger quantity of RNA than the samples would allow. Therefore, the better alternative would be to use real-time PCR because it amplifies the small amount of RNA available from each sample.

Real-time PCR was chosen over conventional PCR due to its convenience to retrieve the results. Having to prepare for reverse transcription PCR and then detecting the PCR products in an agarose gel is cumbersome. The PCR products may also degrade before it could be detected in the gel. Furthermore, the intensity of the gel bands can be subjective to the viewer. By using real-time PCR, all the possible problems faced when using conventional PCR could be eliminated.

Real-time PCR works on the principle of heat amplification and fluorescence detection [Applied Biosystems]. Probes that are tagged with fluorescent dyes are placed together with the necessary constituents, such as enzymes, magnesium chloride and dNTP (deoxyribonucleotides). During DNA amplification, the probes bound to the newly synthesized DNA release the fluorophores, allowing them to fluoresce [Applied Biosystems]. The total fluorescence intensity is captured by a detector in the real-time PCR machine after every cycle and is represented as a logarithmic value which is plotted on a graph [Applied Biosystems]. Thus, the amount of fluorescence is directly proportional to the quantity of PCR products. By studying the graph plotted, the concentration of DNA amplicons and the Ct values can be known without proceeding to other protocols. If the Ct value is low, it would mean that there was a high concentration of DNA to begin with, hence the fluorescence threshold can be exceeded quite rapidly [University of South Carolina, School of Medicine]. However, if the Ct value is higher, it would mean that the concentration of DNA was lower. Applying this into the context of the project, a higher Ct value would mean a better inhibition of EV71 replication and a lower Ct value would mean a poor inhibition of EV71.

If reverse transcription real-time PCR was to be carried out, the probe chemistry to be used would be TaqMan probe, a product of Applied Biosystems [CyberGene AB]. Other probe chemistries commonly used for real-time PCR detection are SYBR Green I and Hybridization probe. SYBR Green I is not ideal because it is rather unspecific. SYBR Green I is a fluorescence dye that was previously used in DNA gel electrophoresis to detect double-stranded DNA bands in the gel [Roche Applied Science]. It detects double-stranded DNA by binding to the minor grooves of the DNA double-helix structure

[Roche Applied Science]. This dye's ability to bind to double-stranded DNA makes it unspecific because it binds to all double-stranded DNA. If a mismatch takes place in during PCR, SYBR Green I would still detect it and add it to the fluorescence graph. Hybridization probe chemistry operates on the principle of fluorescence resonance energy transfer (FRET) [Premier Biosoft International]. Two probes, which are complementary to the template and located side-by-side when bound to the template, each contains a dye. One of the probes would be bound to a donor dye while the other has an acceptor dye [Premier Biosoft International]. During the annealing phase of the PCR, the two probes would bind to the template DNA strand and during the extension phase, the polymerase would hydrolyze the two templates. Once the probes are in close proximity, ultraviolet excitation causes the donor dye to transfer energy to the acceptor dye [Premier Biosoft International]. In order to return to the rest stage, the acceptor dye would have to release the energy received from the donor dye, and the energy is released in the form of fluorescence [Premier Biosoft International]. The fluorescence can then be captured by the fluorescence detector. Hybridization probe is accurate owing to the fact that the two probes are highly specific; the dyes will only work if the probes are accurately bound to the template [Premier Biosoft International]. The disadvantage of using hybridization probe is that it requires two dyes on two separate probes. If the concentration of probes used was high, the distance between the probe molecules would be closer and may trigger inappropriate fluorescence. This might lead to the high background fluorescence or unaccepted small peaks in the fluorescence graph.

TaqMan probe is also based on the principle of FRET [Premier Biosoft International]. Unlike the hybridization probe, it uses only one probe for fluorescence to take place. It also uses two dyes but instead of donor and acceptor dyes, it uses a quencher dye and a reporter dye [Premier Biosoft International]. Hence, unlike the hybridization probe which requires the two dyes to be in close proximity to emit fluorescence, no fluorescence is emitted when the quencher and reporter dyes are in close proximity [Premier Biosoft International]. However, TaqMan probe is an oligonucleotide, like the probes in the Hybridization chemistry. The commonly-used reporter dye, 6-carboxy-fluorescein (FAM), is located at the 5' end of the probe and the quencher dye, 6-carboxytetramethyl-

rhodamine (TAMRA), is located at the 3' end of the probe [CyberGene AB]. Separated only by the length of the oligonucleotide, the quencher dye suppresses the fluorescence from the reporter dye. During the annealing phase of the PCR, the polymerase hydrolyzes the probe and the two dyes are released from the probe [Premier Biosoft International, CyberGene AB]. Now being apart from each other, the reporter dye is no longer quenched and the fluorescence is emitted, which is detected and quantified [Premier Biosoft International]. This is repeated every cycle of the annealing phase and does not interfere with the accumulation of PCR products [Premier Biosoft International].

6.3 Therapeutic Studies

Having proven that the three lengths of siRNAs were able to inhibit EV71-induced CPE, the next step would be to test if similar effects could be seen in therapeutic studies. The study conducted showed that siRNA was able to inhibit viral replication in a prophylactic system. Therapeutic studies are important because infection is to be carried out before siRNA is transfected. An analogy to describe the importance of therapeutic studies would be the events that take place when experiencing an illness. Normally, medication is not taken until the symptoms of an illness begin to show. Similarly, if the cells were to be infected before transfection with siRNA, would the RNAi pathway still be able to inhibit viral replication? To answer this question, prophylactic studies could be carried out and inhibition detection assays such as western blot, plaque assay and real-time PCR could be used as means of measuring the effectiveness of inhibition of EV71 replication.

Therapeutic studies could be carried out using the same methodology as the prophylactic studies, except that infection would take place before the transfection step. Following the timeline shown in Figure 7, infection of the cells using the viral titre determined by plaque assay would commence after the RD cells had been seeded for 24 hours. The cells would be infected for 24 hours before being transfected with the three different siRNAs at different concentrations. In the therapeutic studies, the serum starvation step would be omitted. Serum starvation was carried out in the prophylactic studies because it weakened the cells, making them more receptive to the transfection agent carrying the siRNA after

being starved for several hours. Serum starvation is omitted in the therapeutic studies because the cells will be weakened by EV71 infection and be receptive to the transfection agent carrying the siRNA. Cells may be stained 24 or 48 hours post transfection for cell viability plaque assay or harvested for western blot or reverse transcription real-time PCR.

6.4 *In vivo* Studies

If the therapeutic studies are able to show signs of recovery for the RD cells, *in vivo* studies may be carried out as well. A previous study using 19mer siRNA to trigger the RNAi pathway in mice infected with EV71 was shown to be successful [Tan *et al*., 2007]. The 19mer siRNA used in that study was targeted at the 3D polymerase region, which is the same as the one targeted in this project. Besides using siRNA, the group also used short hairpin RNA (shRNA) [Tan *et al*., 2007]. The shRNA, instead of being comprised of two strands of RNA, is made of a single stranded RNA which back folds to bind complementarily to itself, thus forming a hairpin structure. Using that study as a guideline, *in vivo* studies could be replicated using the three siRNAs used in this project.

Newborn mice that were a few days old, suckling mice, could be used for prophylactic testing. In the previous study, the suckling mice were first injected with 10nM of 19mer siRNA before being infected with EV71 virus [Tan *et al*., 2007]. Within a few days, the infected mice that did not receive siRNA showed signs of acute flaccid paralysis, suffered significant weight loss and died one to two weeks post infection [Tan *et al*., 2007]. In contrast, the mice that received siRNA and were infected did not show signs of weight loss [Tan *et al*., 2007].

Using that study as a guideline, the inhibitory effects of the three siRNAs can be tested. There are two angles to test the effectiveness of siRNA against EV71. The previous study had shown that siRNA were able to inhibit EV71 replication but shRNA were unable to [Tan *et al*., 2007]. With this finding, the three siRNAs to be used stand a good chance against EV71 replication. So, one of the angles to approach the test is to put 27mer and

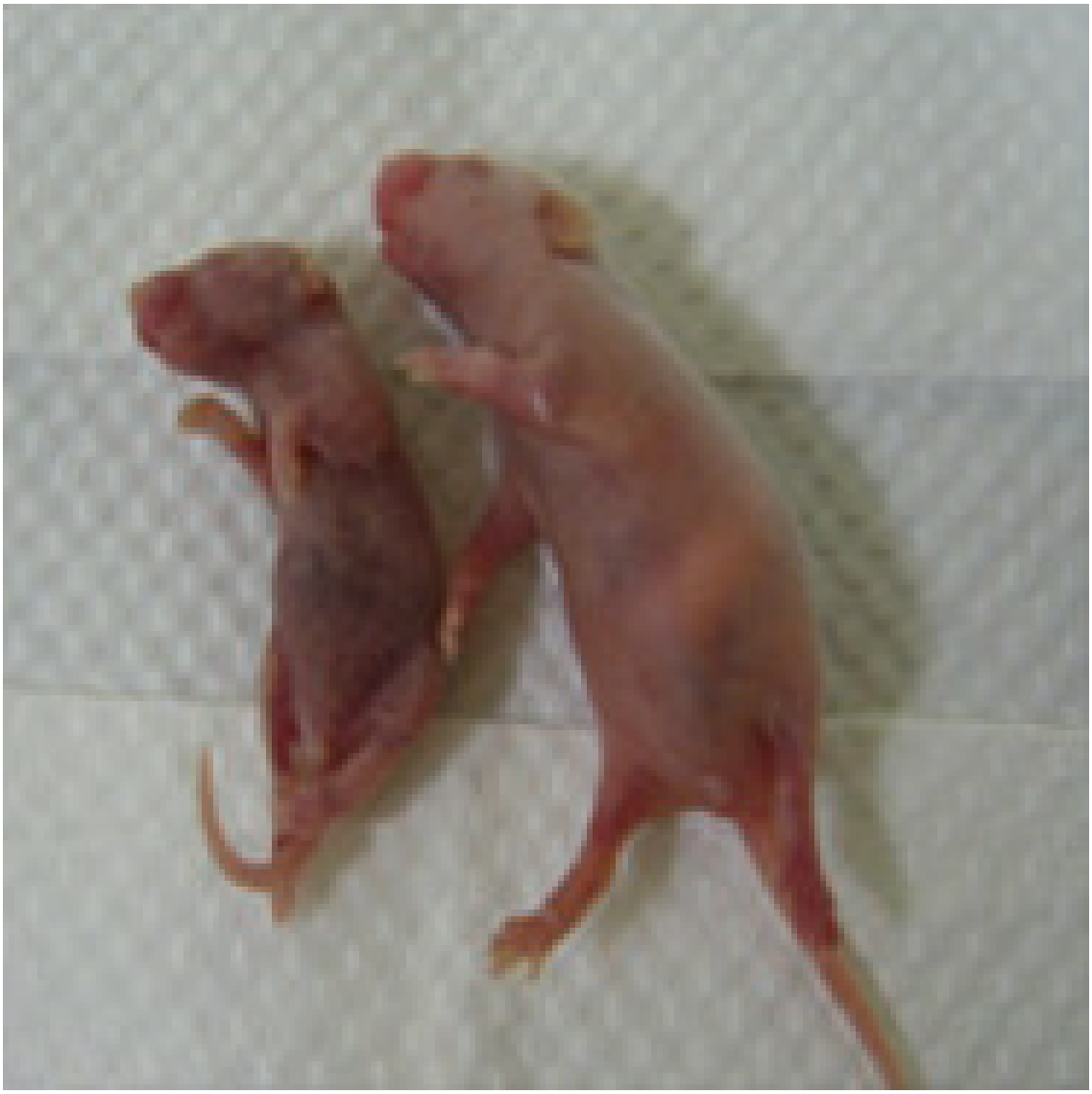


Figure 12: EV71-infected mice

The picture above shows two mice that were used in the study conducted by Tan E.L. *et al*. The mouse on the left was infected with EV71 and suffered severe weight loss and hind limb paralysis. The mouse on the right was not infected. [Tan E. L. *et al*., 2007]

29mer, both of which were better at inhibiting EV71 replication than 19mer *in vitro*, to the test to see if they can still exhibit better inhibitory effects than 19mer *in vivo*.

EV71 infection. In the research conduct by Tan E.L. *et al*., 19mer siRNA was proven effective in the inhibition of EV71 infection [Tan *et al*., 2007]. For the future development of this project, 27mer and 29mer, which were proven to be more effective than 19mer *in vitro*, can be tested *in vivo* to determine whether it is still more effective than 19mer.

Another approach to the *in vivo* studies is to test for concentration dependency. In the *in vivo* system, there are more factors to consider. One of the factors that can affect the mice is their immune system. Certainly after being infected with the virus, the immune system of the mice would begin to fight off the infection through activation of the B and T lymphocytes, natural killer (NK) cells, interferon pathways and antigen presentation. These immune responses together with symptoms such as fevers could ultimately destroy the siRNA. The cytotoxic effects of the transfection agent, Lipofectamine™ 2000, and the siRNA could induce more damage to the cells than intended. However, the results of the previous research showed that the mice were able to withstand the cytotoxicity of the siRNA and the transfection agent, and able to survive the EV71 infection [Tan *et al*., 2007].

For the *in vivo* studies, mice that are about three days old are to be used. As discussed and researched by Yu *et al*., mice that were six days old had developed better immunity against EV71 and were suffered no mortality as compared to the former, thus it would not be suitable to use older mice [Yu *et al*., 2000]. This finding correlates with the reactions seen in human hosts. As mentioned in the background, children that are below the age of five are more susceptible to EV71 infection than older children and adults. Therefore, it is important to use neonatal mice for *in vivo* studies because it can simulate as closely as possible to the situation in human hosts.

Besides mice, it was shown by Hashimoto *et al*. in 1982 that another animal that is susceptible to EV71 is the cynomolgus monkey [Hashimoto *et al*., 1982]. However, it would more inconvenient to use monkeys than mice because monkeys are more expensive and more cumbersome to maintain than mice. Experienced with animals, it would be easier to start with smaller animals that are easier to handle.

If given more time and funding, deeper research of this project and the tests explained in this section could lead to the identification of a potential drug to fight against EV71 and possibly save the lives of many children.

7. Conclusion

EV71 is one the major causes for HFMD due to its ability to cause severe neurological symptoms and death in children [Chong *et al*., 2005]. Since its discovery, EV71 had caused outbreaks in many countries and more recently, the Asia-Pacific regions [Ho *et al*., 1999; Melnick, 2004]. Patients infected with EV71 are at risk for irreversible neurological symptoms, pulmonary oedema and haemorrhage apart from the common symptoms of HFMD, such as ulcers in the oral cavity, fevers, and exanthema on the palms and feet [Melnick, 2004; Singh *et al*., 2002; Sarnow, 2003; Hellen, 1995].

Current treatments do not target EV71 itself but rather the symptoms displayed by the victims. However, promising drugs have been found and are undergoing clinical trials. One of the most promising drugs is pleconaril, which was used to treat colds caused by Rhinovirus, a group of viruses that are in the same family as EV71, Picornavirus [Romero, 2001]. Another drug that was shown to be effective against EV71 is aloe-emodin, which can be found in Chinese herbs [Lin *et al*., 2008]. Aloe-emodin combats EV71 infection by up-regulating interferons and antiviral activities [Lin *et al*., 2008].

RNAi is a pathway that is widely researched since its discovery due to it potential to be an antiviral therapy. It is triggered by siRNA no longer than 30 nucleotides. This project tested the ability of RNAi to inhibit EV71 using different concentrations and different lengths of siRNAs targeting the 3D polymerase of the virus. In a prophylactic system, RD cells were transfected with siRNA and then infected with EV71. Cell viability plaque assay and western blot were conducted to demonstrate the inhibitory effects of the siRNAs at different concentrations. The results proved that the siRNAs are able to inhibit EV71-induced CPE to different degrees based on the concentration of siRNA transfected, with 27mer and 29mer siRNAs having the best inhibitory effects.

This project has shown that RNAi does indeed have the potential to be used as an antiviral therapy. RNAi is favoured because the area of target can be customized to

silence a particular gene. If further developments can be carried out, the three siRNAs could be established to be the key to fighting EV71.

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9. Appendices

Appendix I

Protocol for culture media

MEM (1X)	450mL
Foetal bovine serum	50mL
Penicillin/Streptomycin (1X)	1mL
Total	501mL

Appendix II

EV71 Genome Sequence

```
Sequence 7411 BP; 2000 A; 1766 C; 1794 G; 1851 T; 0 other;
ttaaaacagc tgtgggttgt acccactcac agggcccacg tggcgctagc actctggttc        60
tgcggaacct ttgtgcgcct gttttacgcc cgccccccaa tttgcaactt agaagcaata       120
cacaacactg atcaacagca ggcatggcgc accagctatg tcttgatcaa gcacttctgt       180
ttccccgggc cgagtatcaa tagactgttc acgcggttga aggagaaagc gcccgttatc       240
cggctaacta cttcgagaaa cctagtagca ccattgaagc tgcagagtgt ttcgctcggc       300
acttcccccg tgtagatcag gtcgatgagt cactgcaatc cccacgggcg accgtggcag       360
tggctgcgct ggcggcctgc ctatggggca acccatagga cgctctaatg tggacatggt       420
gcgaagagtc tattgagcta gttagtagtc ctccggcccc tgaatgcggc taatcctaac       480
tgtggagcac atgccttcaa tccagagggt agtgtgtcgt aatgggcaac tctgcagcgg       540
aaccgactac tttgggtgtc cgtgtttcct tttatcttta cattggctgc ttatggtgac       600
gattatagaa ttgttaccat atagctattg gattggccat ccggtgtgca atagagctat       660
tatatacctg tttgttggct ttgtaccact aaccttaaaa tctataacca ccctcgattt       720
tatattaacc ctcaatacaa tcaaacatgg gctcacaggt gtctactcag cgatccggct       780
cccacgagaa ctccaattca gctacagaag gctccaccat taattacact accatcaact       840
attacaaaga ctcctatgct gcgacagcgg gcaaacagag cctcaagcaa gaccctgata       900
aatttgctaa ccctgtcaag gacattttca ctgaaatggc tgcaccactg aagtctccat       960
ccgctgaggc ttgtggttac agtgatcgcg tggcacaact caccattgga aactccacca      1020
tcactacaca ggaggcggcg aatatcatag tcggttatgg tgagtggccc tcatactgct      1080
ctgatgacga tgctacagcg gtggacaagc caacgcgccc agatgtttca gtgaataggt      1140
tttatacgtt ggacactaaa ttgtgggaaa agtcatccaa ggggtggtat tggaagtttc      1200
ctgatgtact gactgagacc ggagtctttg gccagaatgc acagtttcac tatttatata      1260
ggtcaggatt ttgcattcat gtgcaatgta atgctagcaa gttccatcaa ggagcgttgt      1320
tagtcgctat acttccagag tatgttatag ggacagtggc aggcggcaca ggaactgagg      1380
acagccaccc tccttacaaa caaacacaac ctggcgccga tggttttgag ttgcagcacc      1440
cgtacgtact cgatgctggg attcctatat cacaattaac agtgtgcccc caccaatgga      1500
ttaacctacg gaccaataac tgtgccacaa taatagtgcc gtatatgaac acactgcctt      1560
tcgactctgc cctgaaccat tgcaactttg ggctgttggt ggtgcccatt agcccactag      1620
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cccagtggtc aggatcactg gaggttactt ttatgttcac cgggtctttc atggccacgg      2100
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caatgctggg cacacatgtt atctgggatt ttgggctaca atcatctgtc acccttgtaa      2220
taccatggat cagcaacacc cactacagag cgcatgcccg ggatggagtg ttcgattact      2280
ataccacagg actggttagt atctggtatc aaacaaacta cgtggttcca attggggcac      2340
ccaacacagc ttacataata gcactagcgg cagcccagaa gaatttacc atgaaactgt       2400
gtaaagacac cagtcacata ttacagacag cctctattca gggagataga gtggcagatg      2460
tgatagagag ctctatagga gatagtgtga gtagggcact tacccaggcc ctgccagctc      2520
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tccaagctgc tgaaataggg gcatcgtcaa atactagtga tgagagtatg attgagacac      2640
gatgcgttct taattcacac agtacggcag agaccaccct ggacagcttc ttcagtaggg      2700
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ctaattggga tatagacata actggttacg cacaaatgcg caggaaagtg gagctgttca      2820
cctacatgcg ctttgatgcg gaattcactt ttgttgcgtg cactcctact ggtgaggttg      2880
tcccacaatt acttcagtat atgtttgttc cccctggtgc tcccaaacca gagtctagag      2940
aatcacttgc ttggcagaca gccacaaacc cctcagtttt tgtcaagttg actgatcccc      3000
```

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tggttgtcag gatatatatg agaatgaagc atgtcagggc gtggatacct cgcccgatgc      3240
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```

Appendix III

Layout for plaque assay (determination of viral titre)

Viral Dilution Factor: 10	Viral Dilution Factor: 10	Viral Dilution Factor: 10	Viral Dilution Factor: 10	Viral Dilution Factor: 10	Viral Dilution Factor: 10
Viral Dilution Factor: 100	Viral Dilution Factor: 100	Viral Dilution Factor: 100	Viral Dilution: 100	Viral Dilution: 100	Viral Dilution: 100
Viral Dilution: 1000 - 70 -	Viral Dilution: 1000	Viral Dilution: 1000	Viral Dilution: 1000	Viral Dilution: 1000	Viral Dilution: 1000
Viral Dilution: 10,000	Viral Dilution: 10,000	Viral Dilution: 10,000	Viral Dilution: 10,000	Viral Dilution: 10,000	Viral Dilution: 10,000

Protocol for CMC Agar

1. 5 grams of CMC powder was dissolved in 250mL of distilled water.
2. The mixture was autoclaved to facilitate complete dissolving.
3. 750mL of culture media was added and mixed gently to create 2X CMC agar

Appendix IV

Transfection and Infection Layout for Cell Viability Plaque Assay

19mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL	19mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL	27mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL	27mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL	29mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL	29mer 3nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 150uL (incubate) + OptiMEM – 248.8uL
19mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL	19mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL	27mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL	27mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL	29mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL	29mer 5nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 2.5uL (incubate) + OptiMEM – 396.3uL
19mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL	19mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 19mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL	27mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL	27mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 27mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL	29mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL	29mer 10nM Lipofect – 1.2uL OptiMEM – 100uL 29mer siRNA – 5uL (incubate) + OptiMEM – 393.8uL
19mer Scr 10nM Lipofect – 1.2uL OptiMEM – 100uL 19mer Scr siRNA – 5uL (incubate) + OptiMEM – 393.8uL	29mer Scr 10nM Lipofect – 1.2uL OptiMEM – 100uL 29mer Scr siRNA – 5uL (incubate) + OptiMEM – 393.8uL	Positive control No infection	No transfect Only infect	Transfection Infection Lipofect – 1.2uL OptiMEM – 100uL (incubate) + OptiMEM – 398.8uL	Transfection No infection Lipofect – 1.2uL OptiMEM – 100uL (incubate) + OptiMEM – 398.8uL

Appendix V

Transfection and Infection Layout for Western Blot

siRNA

3nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 7.5uL (incubate) + OptiMEM – 1986.5uL	5nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 12.5uL (incubate) + OptiMEM – 1981.5uL	10nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 25uL (incubate) + OptiMEM – 1969.0uL
3nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 7.5uL (incubate) + OptiMEM – 1986.5uL	5nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 12.5uL (incubate) + OptiMEM – 1981.5uL	10nM Lipofect – 6.0uL OptiMEM – 500uL siRNA – 25uL (incubate) + OptiMEM – 1969.0uL

Controls

19mer Scr 10nM Lipofect – 6.0uL OptiMEM – 500uL 19mer Scr siRNA – 25uL (incubate) + OptiMEM – 1969.0uL	Positive control No infection	Transfection Infection Lipofect – 6.0uL OptiMEM – 500uL (incubate) + OptiMEM – 1994.0uL
29mer Scr 10nM Lipofect – 6.0uL OptiMEM – 500uL 29mer Scr siRNA – 25uL (incubate) + OptiMEM – 1969.0uL	Transfection only Lipofect – 6.0uL OptiMEM – 500uL (incubate) + OptiMEM – 1994.0uL	No transfect Infection only

Appendix VI

Student's T Tests

(a)

t-Test: Two-Sample Assuming Equal Variances

	19.3	*19.5*
Mean	17.5	8
Variance	4.333333	2.666667
Observations	4	4
Pooled Variance	3.5	
Hypothesized Mean Difference	0	
Df	6	
t Stat	7.181325	
P(T<=t) one-tail	0.000184	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.000368	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 19.3 and 19.5

(b)

t-Test: Two-Sample Assuming Equal Variances

	19.5	*19.10*
Mean	8	4
Variance	2.666667	3.333333
Observations	4	4
Pooled Variance	3	
Hypothesized Mean Difference	0	
Df	6	
t Stat	3.265986	
P(T<=t) one-tail	0.00856	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.01712	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 19.5 and 19.10

(c)

t-Test: Two-Sample Assuming Equal Variances

	19.3	*19.10*
Mean	17.5	4
Variance	4.333333	3.333333
Observations	4	4
Pooled Variance	3.833333	
Hypothesized Mean Difference	0	
Df	6	
t Stat	9.751254	
P(T<=t) one-tail	3.34E-05	
t Critical one-tail	1.94318	
P(T<=t) two-tail	6.69E-05	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 19.3 and 19.10

(d)

t-Test: Two-Sample Assuming Equal Variances

	27.3	*27.5*
Mean	14	4.5
Variance	3.333333	1.666667
Observations	4	4
Pooled Variance	2.5	
Hypothesized Mean Difference	0	
df	6	
t Stat	8.497058	
P(T<=t) one-tail	7.27E-05	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.000145	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 27.3 and 27.5

(e)

t-Test: Two-Sample Assuming Equal Variances

	27.5	*27.10*
Mean	4.5	2
Variance	1.666667	1.333333
Observations	4	4
Pooled Variance	1.5	
Hypothesized Mean Difference	0	
df	6	
t Stat	2.886751	
P(T<=t) one-tail	0.013906	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.027811	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 27.5 and 27.10

(f)

t-Test: Two-Sample Assuming Equal Variances

	27.3	*27.10*
Mean	14	2
Variance	3.333333	1.333333
Observations	4	4
Pooled Variance	2.333333	
Hypothesized Mean Difference	0	
df	6	
t Stat	11.10984	
P(T<=t) one-tail	1.58E-05	
t Critical one-tail	1.94318	
P(T<=t) two-tail	3.17E-05	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 27.3 and 27.10

(g)

t-Test: Two-Sample Assuming Equal Variances

	29.3	*29.5*
Mean	14	3.5
Variance	0.666667	0.333333
Observations	4	4
Pooled Variance	0.5	
Hypothesized Mean Difference	0	
df	6	
t Stat	21	
P(T<=t) one-tail	3.8E-07	
t Critical one-tail	1.94318	
P(T<=t) two-tail	7.6E-07	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 29.3 and 29.5

(h)

t-Test: Two-Sample Assuming Equal Variances

	29.5	*29.10*
Mean	3.5	1
Variance	0.333333	0.666667
Observations	4	4
Pooled Variance	0.5	
Hypothesized Mean Difference	0	
df	6	
t Stat	5	
P(T<=t) one-tail	0.001226	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.002452	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 29.5 and 29.10

(i)

t-Test: Two-Sample Assuming Equal Variances

	29.3	*29.10*
Mean	14	1
Variance	0.666667	0.666667
Observations	4	4
Pooled Variance	0.666667	
Hypothesized Mean Difference	0	
df	6	
t Stat	22.51666	
P(T<=t) one-tail	2.51E-07	
t Critical one-tail	1.94318	
P(T<=t) two-tail	5.02E-07	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between the 29.3 and 29.10

(j)

t-Test: Two-Sample Assuming Equal Variances

	19.10	*27.10*
Mean	4	2
Variance	3.333333	1.333333
Observations	4	4
Pooled Variance	2.333333	
Hypothesized Mean Difference	0	
df	6	
t Stat	1.85164	
P(T<=t) one-tail	0.056766	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.113532	
t Critical two-tail	2.446912	

more than 0.05, test is non significant
there is no significance between 19.10 and 27.10

(k)

t-Test: Two-Sample Assuming Equal Variances

	27.10	*29.10*
Mean	2	1
Variance	1.333333	0.666667
Observations	4	4
Pooled Variance	1	
Hypothesized Mean Difference	0	
df	6	
t Stat	1.414214	
P(T<=t) one-tail	0.103516	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.207031	
t Critical two-tail	2.446912	

more than 0.05, test is non significant
there is no significance between 27.10 and 29.10

(l)

t-Test: Two-Sample Assuming Equal Variances

	19.10	*29.10*
Mean	4	1
Variance	3.333333	0.666667
Observations	4	4
Pooled Variance	2	
Hypothesized Mean Difference	0	
df	6	
t Stat	3	
P(T<=t) one-tail	0.012004	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.024008	
t Critical two-tail	2.446912	

less than 0.05, test is significant
there is significance between 19.10 and 29.10

(a-c) comparison between concentrations for 19mer

(d-f) comparison between concentrations for 27mer

(g-i) comparison between concentrations for 29mer

(j-l) comparison between siRNAs at 10nM