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Functional characterization of coxsackievirus A9-eGFP vector

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Picornaviruses are small, single-stranded, positive-sense, and non-enveloped RNA viruses, that cause various diseases in humans and mammals. More than 300 human picornavirus types are identified as responsible for diseases from respiratory illness to life-threatening CNS disease, flaccid paralysis, and meningitis. Even though there are identified picornavirus types, there are no direct antiviral drugs against this virus, and barely any vaccines are available. RNA viruses like picornaviruses are prone to mutations that alter the virus tropism and pathogenesis, which complicate the development of therapeutic strategies. For picornavirus research, viral cDNA clones or viral vectors research is necessary, and the standard methods used for developing cDNA clones however are old-fashioned and complex. In cancer therapy, picornaviruses show promise as vectors for delivering therapeutic materials to affected cells to destroy the cells completely.

The project aims to characterize the functionality of the CVA9-eGFP virus vector in cell culture by measuring its infection efficiency, replication dynamics, the expression of the eGFP marker gene, and the potentiality of these vectors for cancer therapy. The T7-promoter-based system was used for the in vivo rescue of CVA9 and CVA9-eGFP virus particles from the viral cDNA or T7-promoter-tagged PCR amplicons. An optimized long-PCR protocol utilized for successful amplification of the full-length CVA9 viral genome with T7-promoter for virus rescue using the T7 RNA polymerase (T7RNAP) system. Moreover, different promoters, such as T7 and CMV promoters' efficiency for virus rescue and viral replication were evaluated.

The experiment involved several steps from transforming the E. coli with plasmid vectors, isolating and purifying different CVA9 and CVA9-eGFP DNA, verification through restriction digestion and agarose gel electrophoresis, generating T7-promoter-tagged PCR amplicons, transfecting mammalian cells, and eventually observing the infected cells using an EVOS FL AUTO fluorescence microscope.

CVA9-eGFP PCR amplicons successfully transfect the BHK-21 and T7-BSR cells which generate infectious virus particles indicating that the CVA9-eGFP vectors function properly within mammalian cells. Addressing the fundamental research questions and testing the hypothesis, this research gives us valuable insights into the functionality and behaviour of the CVA9-eGFP vector. Using the information from this research in molecular virology fields offers a foundation for developing novel therapeutic properties against picornavirus diseases and cancer.

Keywords: picornavirus, CVA9-eGFP, virus vector, reverse genetics, long-PCR, T7 RNA polymerase.

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1 Introduction

Picornaviruses are small, non-enveloped RNA viruses whose genome size ranges from 6.5 kb to 8.5 kb, and consist of a single-stranded, positive-sense RNA molecule. This virus family causes various diseases in humans and mammals. Enteroviruses and rhinoviruses are representatives of picornaviruses that are very common and infect humans. The most common disease caused by picornaviruses is the common cold, often with mild symptoms. However, picornaviruses may also cause serious diseases like flaccid paralysis and meningitis. There are no direct antiviral drugs against this virus, and there are barely no vaccines available for these viruses. As RNA virus picornaviruses are very much susceptible to mutation, and thus it is hard to develop targeted antiviral drugs with long-lasting impact.

Genetic manipulation for generating modified picornaviruses has opened up new doors for studying and understanding viral properties, including viral behaviour, disease mechanisms, viral transmission, pathogenicity, and finally therapeutic interventions. Some picornaviruses have oncolytic properties, which make them ideal candidates in cancer therapy development. Those picornaviruses can recognize specific cell surface receptors and selectively infect and kill those over-expressed cancer cells while sparing the healthy cells. Viral vectors generated from picornaviruses can also be used for delivering specific therapeutic agents to the targeted cells making them potential tools in vector-based therapies.

Introducing marker genes, such as the green fluorescent protein (eGFP) encoding genes into the viral genomes makes it possible to track and visualize viral infection and its replication within cells in real-time by using fluorescence microscopes. Successful cell transfection is crucial for rescuing viruses, and optimized transfection strategies need to be developed for using these viral vectors for delivering therapeutic agents. Different strong promoters, such as T7-promoter or CMV-promoter-based systems can be used for enhancing viral RNA transcription and successful replication. The T7-promoter can utilize the T7 RNA polymerase enzymes produced in T7-BSR cells.

There has been a significant advancement in reverse genetics, which accelerates the picornaviruses vector research. Reverse genetics techniques allow the generation of functional infectious virus particles from cDNA clones, which enables genetic modifications and analysis of their effects on viral functionality. By using these techniques researchers can modify the

picornavirus to develop efficient viral vectors for delivering antiviral therapeutic properties to kill cancer cells.

1.1 Picornavirus taxonomy

Picornaviruses are small RNA viruses that belong to the family *Picornaviridae* within the order *Picornavirales*. There are 68 genera containing 158 species and hundreds of virus types at the time of writing (20/06/24). More species are under review for including the Picornaviridae family for classification (Zell et al., 2017) (International Committee on Taxonomy of Viruses Executive Committee, 2020). In this project, the Griggs strain of Coxsackievirus A9 (CVA9) was used which belongs to the *Enterovirus* genus. More species are under review for including in the *Picornaviridae* family for classification ((Zell et al., 2017), https://www.picornaviridae.com/). The *Enterovirus* genus has a large variety of virus types, including 15 species: *Enterovirus A* through *L* and *Rhinovirus A* to *C*. Coxsackievirus A9 (CVA9) parts of this *Enterovirus B* species. CVA9 taxonomy can be presented in this way: Order: *Picornavirales*, Family: *Picornaviridae*, Genus: *Enterovirus*, Species: *Enterovirus B*, Type: Human coxsackievirus A9, Isolate: Griggs strain.

1.2 Structure of picornavirus particles

Picornavirus particles are small non-enveloped viruses, built with single-stranded, positive-sense RNA genomes. The virus particle's outer diameter is between 30 to 32 nm and an icosahedral protein capsid encloses the whole genome. The capsid of picornaviruses is made up of 60 protomers (small building blocks) and these protomers are formed by all four distinct structural proteins: VP1, VP2, VP3, and VP4. From V1-3 proteins formed the outer shell of the capsid, on the contrary, protein VP4 is found on the inner surface of the shell making them invisible in the image (J et al., 2016). When the VPs cluster together triangular protomers are formed. 5 protomers are grouped to make pentamers, and twelve pentamers come together to complete the icosahedral capsid structure (Zell et al., 2017). Figure 1 shows the structure of picornaviruses.

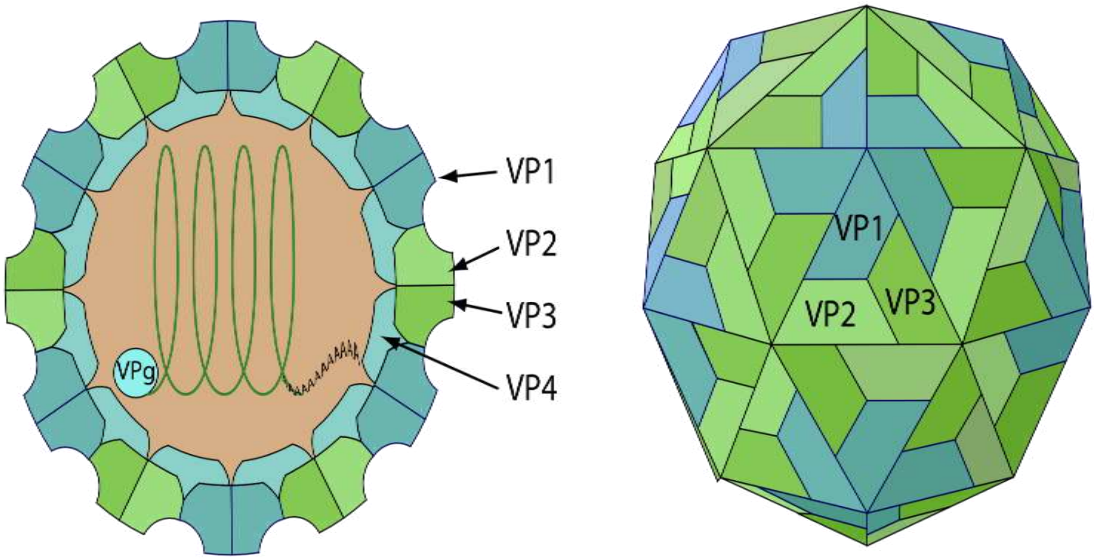

Figure 1 Structure of Picornavirus. The right-side image showed the atomic model of the picornavirus capsid. The schematic representation of a picornavirus particle is depicted in the first 2 images. The single-stranded, positive-sense naked RNA genome is encapsulated by a non-envelop, icosahedral protein capsid. The capsid shell is made up of 60 protomers, where VP1, VP2, and VP3 construct the outer shell of the capsid and VP4 is buried inside the capsid. The VP1, VP2, and VP3 capsid proteins share a common core structure of eight β-strands to form protomers, which later form pentamers. These pentamers finally form the icosahedral structure of the capsid. (Figure edited from ViralZone, SIB Swiss Institute of Bioinformatics).

During the maturation process of picornavirus virion, the VP0 capsid protein is cleaved into VP2 and VP4 components in the final stage. However, in some picornaviruses such as in Parechoviruses development periods, the VP0 protein remains intact and the mature virion shells contain only VP0, VP1, and VP3 structural proteins in the capsid (Nateri et al., 2000). The VP1, VP2, VP3, and VP4 structural proteins have different sequences and are not homologous to each other, whereas the VP4 protein is the most different from other proteins. If we look into the poliovirus VP protein sequences, VP1 consists of 306 amino acids (~33kDa), VP2 is 272 (~30kDa) amino acids, VP3 is 238 amino acids (~26kDa), while VP4 contains only 69 amino acids (~7.5 kDa) (Tuthill et al., 2010). Although these VP1-3 capsid proteins have different amino acid lengths and molecular weights, they share a common basic structure where they are composed of an arrow-like eight-stranded β-barrel structure, interconnected by loops of different lengths. The orientation of β-barrels and different loop lengths influence the binding specificity of the virus receptor by determining the surface topology of the virus particles.

1.3 Genomic composition of picornavirus

The genomic composition of Picornaviridae is compact and genetic material is well-designed. It has a single-stranded, positive-sense RNA, consisting of 6500 to 8500 nucleotides in length. This positive-sense RNA when once inside the host cell, the RNA acts like a messenger RNA (mRNA) (Cifuente and Moratorio, 2019). This means the presence of an internal ribosomal entry site (IRES) allows a long polyprotein chain to be translated directly from it and then cleaved into smaller proteins that are needed for virus production and virus structure (Racaniello, 2001; Tuthill et al., 2010). Mutation in the genome is common as the 3D RNA polymerase amplifies the genome in a way that is more susceptible to errors compared to the DNA polymerase enzymes. At the 5´ ends of the viral genome, a small genome-linked VPg peptide, which is coded by a single viral gene, lengths vary from 22 to 24 amino acids, is covalently bound (Lin et al., 2009). This VPg peptide acts as a protein primer, attaching the RNA polymerase to initiate viral RNA synthesis and as a control for genome translation (Paul et al., 1998; Paul and Wimmer, 2015). On the other 3´ end of the genome, a poly-A tail terminates the genome providing stability to the genome structure. This poly-A tail is a string of several adenine bases, whose lengths vary depending on the virus (Racaniello, 2001; Tuthill et al., 2010). Both ends of the genome have their untranslational region (UTR) and there is a single open reading frame (ORF) present in the whole genome, which means the genome translates into a single polyprotein. The 5´end UTR region is comparatively long, with about 600-1200 nucleotides, and contains important regulatory elements such as the internal ribosomal entry site (IRES) (Knipe et al., 2001; Tuthill et al., 2010). This IRES is essential for viral mRNA translation initiation and for ribosome attachments (Paul et al., 1998). This IRES depends on several different regulatory proteins for initiating protein synthesis. A single long chain of amino acids, a large polyprotein produced by the single ORF codes, contains lots of information in a small genome. Viral proteases then cut this long protein into smaller individual proteins (Pierce et al., 2023). In that polyprotein, the viral structural proteins (VP1-4) are located in the N-terminal region, while the remaining sections code for non-structural proteins that influence the host cell's condition making it optimal for virus replication (2A-C and 3AD). The position of the structural or non-structural viral protein sequences depends on the specific picornavirus, the structural precursor protein can be immediately after the N-terminal end of

the polyprotein, or a non-structural protein sequence can displace it. Figure 2 explains the enterovirus genome in a box plot.

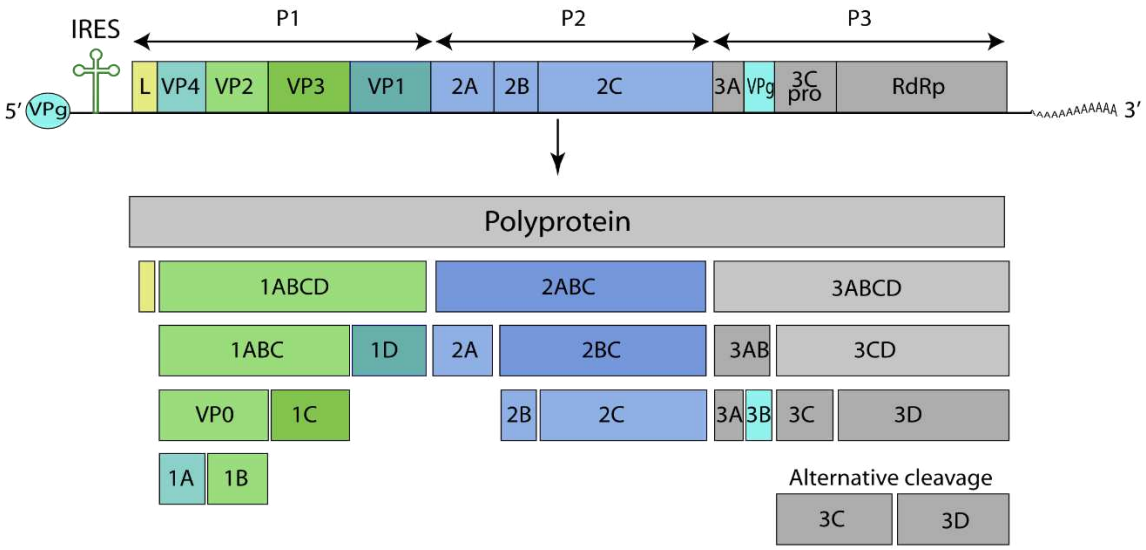

Figure 2 **Organization of a picornavirus genome**. The linear single-stranded RNA genome is approximately 6500-8500 nucleotides in length and can act as an mRNA. The viral RNA genome starts with a viral protein at the 5' end, followed by a long UTR which contains an internal ribosome entry site (IRES). The structural peptides are encoded by the P1 region, while the P2 and P3 regions encode the nonstructural proteins. The genome terminates with a UTR and a poly-A-tail on both ends. The polyprotein encoded by a single ORF gets cleaved by viral proteases into protein products. (The figures taken from ViralZone, SIB Swiss Institute of Bioinformatics)

1.4 Life-cycle of picornavirus

Picornavirus replicates directly in the cytoplasm as the RNA genome acts as mRNA when the virus invades the cell. The cycle starts when the virus attaches to the host cell surface through the cell surface receptors, initiating cell signaling and endocytosis in the host cell (Lin et al., 2009). Picornavirus can use various receptors for their entry, including glycoproteins, integrins, immunoglobulin, and LDLR-like receptors, and this virus-receptor interaction is studied most (Tuthill et al., 2010). In receptor-mediated endocytosis, the virus first binds to the cell-surface receptor, an endocytic vesicle formed which provides access to the virus particle to enter the cytoplasm. The receptor specificity varies between genera and species, and several different endocytosis pathways have been identified. One of the most common endocytosis is called clathrin-mediated endocytosis, where the virus enters the cell through clathrin-coated vesicles. However, this clathrin coat is removed, and later the entry vesicles fused with an early endosome. Another pathway known as caveolin-mediated endocytosis depends on the cell membrane lipid rafts and cholesterols (O'Donnell et al., 2008; Tuthill et al., 2010). After the

virus enters the host cell (endocytically), the viral RNA is released into the cytoplasm, which is called uncoating (Tuthill et al., 2010). The exact principle for this uncoating mechanism is still not clear. However, when the virus is attached to its specific receptor triggers conformational changes in the surface proteins of the viral capsid. These changes in the capsid allow for the formation of a pore in the capsid through the host cell membrane, via these pores viral RNA gains entry to the cytoplasm, and the viral genome uncoated (Shakeel et al., 2013). The released viral RNA is then translated and a polyprotein chain is then synthesized, it's autocatalytically cleaved by viral proteases (mainly by 2Apro and 3Cpro or 3CDpro viral proteinases) creating precursors and functional proteins, important for virus replication and new virus particle production (Lin et al., 2009). The synthesis of RNA occurs in a viral factory, which is a membrane compartment within the cytoplasm that gives protection against the host cells' defence mechanism. Picornavirus utilizes a heterogeneous pool of viral factories, comprising double-membraned vesicles alongside single-membraned spherules and rosettes that are probably generated from the Golgi apparatus, endoplasmic reticulum or from autophagosomes (Netherton and Wileman, 2011). Inside the viral factory, the positive-stranded RNA is copied into a complementary negative-stranded intermediate by the 3D RNA polymerase encoded in the virus, followed by the production of positive-stranded RNA. The new viral capsid formation starts when the VPg linkers of new RNA are assembled into capsids to produce enough capsid proteins from polyproteins. The structural pentamers are accumulated when the coat protein precursor P1 protein produces protomers, eventually forming infectious virus particles inside the host cell (Racaniello, 2001).In the cell model, approximately 5-10 hours are required for a single replication cycle, depending on some variables like the virus type, temperature, pH, host cell, and multiplicity of infection (Racaniello, 2001). After the replication cycle, the picornaviruses are released from the host cell, either through lysis of the cell or in the absence of cytopathic effect using exosomes (Raab-Traub and Dittmer, 2017). The life cycle of picornaviruses is described in Figure 3.

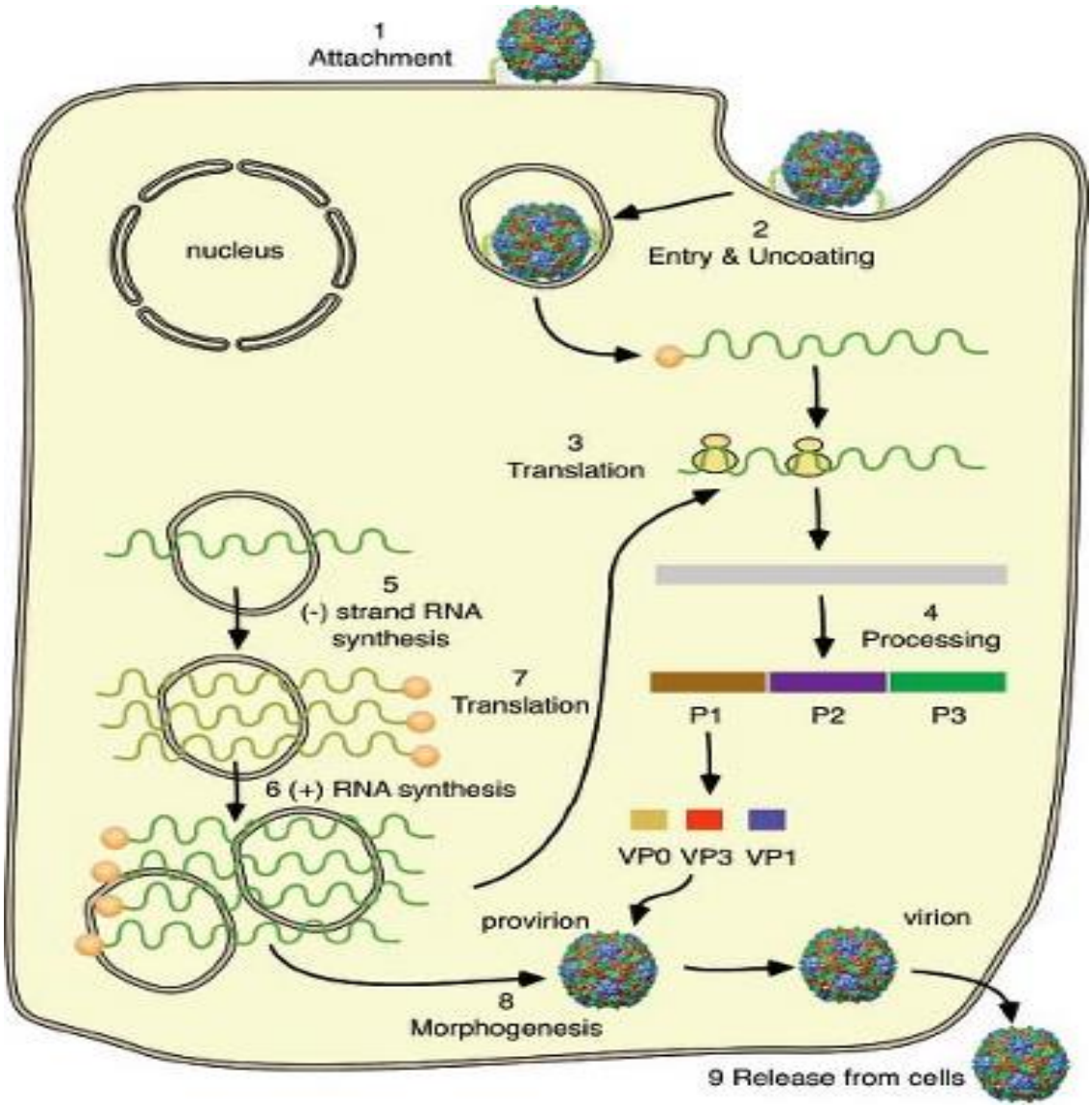

Figure 3 Picornavirus replication cycle. (1) The virus binds to the cell surface receptor, and (2) the virus releases its genome via uncoating. The VPg linker is cleaved from viral RNA, allowing for IRES-dependent translation (3), encoding a polyprotein chain that is cleaved (4) into individual viral proteins. The (+) - stranded RNA is copied into (5) a full-length (-) - stranded RNA by the viral RNA polymerase, which is then copied to produce additional viral proteins (6). Positive-strand RNA is further translated to produce extra viral proteins (7), and later this RNA enters the morphogenetic pathway (8), and finally, the new virus particles come out of the cell through cell lysis (9). Figure edited from (Knipe et al., 2001)

1.5 Clinical manifestation and pathogenicity of picornaviruses

Picornaviruses, cause many clinical symptoms in humans. Most picornavirus infections are asymptomatic. They causes infection from the respiratory tract to hepatitis, polio, meningitis, CNS infection, and flaccid paralysis (Hogle et al., 1985; Rossmann et al., 1985). Multiple species of the *Enterovirus* genus cause diseases with various symptoms developed in patients. One of the world's deadliest enteroviruses throughout history is poliovirus, which causes outbreaks like poliomyelitis and associated paralysis. Rhinoviruses are also common pathogens for infecting human respiratory tracts and developing symptoms of the common cold worldwide (Cifuente and Moratorio, 2019). Certain picornaviruses such as coxsackieviruses cause mild infection with rash and skin lesions, in some cases, they develop severe conditions like myocarditis, meningitis, herpangina, etc. For example, coxsackievirus A9 (CVA9) is responsible for outbreaks of aseptic meningitis (Cui et al., 2010), exanthematous diseases in Japan (Aoki et al., 2012), Hand, foot, and mouth disease, and encephalitis (Cifuente and Moratorio, 2019). Some were also found to be associated with various chronic disease developments. Coxsackievirus B4 is particularly linked to developing type 1 diabetes (T1D) in infected patients (Hyöty, 2016; Jaïdane et al., 2010). However, picornaviruses can cause diseases, and the symptoms mostly develop are mild, subclinical infections are more severe, even fatal infections. Transmission of picornavirus diseases is caused via the fecal-hand-oral or airborne, respiratory routes (Cifuente and Moratorio, 2019). The pathogenicity of the virus depends on several complex factors. The proteins (structural proteins) of the virus outside are attached to the cell's surface receptors and determine which cells can be used for penetration. A single mutation in the epitope's amino acid sequences can significantly impact the virus pathogenicity, i.e. a single amino acid change in the HPeV-1 virus RGD region brought a major change in the infectivity of this virus (Boonyakiat et al., 2001). Within the cell, the replication and spread of infectious virus particles are influenced by the functions of the non-structural proteins and non-coding regions (Lin et al., 2009).

1.6 Prevention against picornavirus infections

It is very hard to treat a picornavirus-infected patient due to the lack of effective treatment methods, and medicines like antiviral drugs and vaccines. To prevent viral diseases from spreading is vaccination in the population, while only a few types of picornavirus vaccines developed. The polio outbreak is world's one of the deadliest outbreaks that happened in the past by poliovirus belonging to the *Enterovirus* genus. The development of the polio vaccine

brought significant change in to fight against this virus diseases. Two types of poliovirus vaccines have been developed in the past: one is Oral OPV vaccines or Sabin vaccines based on their developer, and another one is Injected IPV vaccines (inactivated polio vaccine) also called Salk vaccines according to their manufacturers (Fahmy et al., 2017; Kew et al., 2005; Minor, 1992). In the oral polio vaccine (OPV), all three serotypes of poliovirus virulent factors have been reduced through some modification in the growth environments of the wild types, which leads to the development of virulence-reducing mutations (Minor, 1992). While in IPV vaccines, Formalin inactivates all three different strains of polioviruses. The OPV vaccines, having weakened live viruses, and the IPV vaccines both achieve protection by stimulating a strong humoral immune response in the vaccinated person. Another Enterovirus A71, causes Hand, foot, and mouth disease vaccine developed, yet not been approved by the FDA or EMA, but the research is continuing (Lin et al., 2009). Similar case about antiviral drugs against picornaviruses, there are no registered antiviral drugs available in the market to fight against picornavirus infection compared to the Hepatitis-virus diseases. Pleconaril was an antiviral drug developed to fight against asthma exacerbations and common cold symptoms caused by picornavirus respiratory infection, especially caused by rhinoviruses and enteroviruses (Rotbart et al., 2001). It works by inhibiting viral replication within the host cell, but, it has not been approved for clinical use now ("Pleconaril," 2016). When this drug is taken with other drugs, this drug leads to unpredictable interactions with other drugs and raises safety issues (Ma et al., 2006), so finally, it was removed from the market. Different enviroxime-like compounds and protease inhibitors are used to develop an impactful treatment method, but no established method found yet, which is why the current treatment method is completely symptom-based and supportive care (Rotbart, 2002).

1.7 Picornavirus vectors in therapy

Picornavirus is a single-stranded, positive-sense RNA virus that can be used for treating and preventing diseases due to its therapeutic properties. In gene therapy, a virus can act as a vector for delivering certain genetic material (usually DNA) into a patient's cells. Nowadays, viral vectors are utilized in the treatment of cancer and neurological diseases, alongside the development of viral vaccines. Research has been done with different strains of picornaviruses and enteroviruses showing some significant outcomes, especially with coxsackieviruses and polioviruses. Picornavirus can attach to certain kinds of cell surface receptors using certain parts of their capsid proteins. Picornaviruses, especially enteroviruses can recognize and bind to the overexpressed receptors presented on the cancer cell surface, and after binding it can release its

genomic material into the infected cells for replication, and lead to cell lysis, which makes it a potential Enteroviral tool in cancer therapy researches (McCarthy et al., 2019). Coxsackievirus A21 (CVA21) is widely used for treating various cancers, as it has oncolytic properties that are used in the treatment of melanoma, breast cancer, bladder cancer, and multiple myeloma cancer (Jin et al., 2021). Poliovirus-1 is used as a viral vector because of its ability to induce both humoral and cell-mediated immune responses (Girard et al., 1993). The structural protein VP1 of poliovirus has been modified via small antigenic epitopes inserted from other viruses. For instance, certain epitope proteins are inserted in the capsid of the weakened Sabin poliovirus, and then this modified poliovirus acts as a safe carrier, which stimulates immune cells to produce antibodies that can target the inserted HIV epitopes (Evans et al., 1989). PVS-RIPO is a chimeric oncolytic virus, containing both poliovirus and rhinovirus used to treat certain cancers, such as glioblastoma multiform (GMB) (Beasley et al., 2021), melanoma, and breast cancer treatment.

1.8 Reverse genetics and viral rescue

Reverse genetics systems are powerful tools for understanding the gene(s) functions by analyzing its phenotypic effects after modifying the viral genome sequence. It allows researchers to manipulate the viral genomes by introducing mutations or incorporating reporter genes (Cai and Huang, 2023). In this approach, infectious virus particles are rescued after manipulating the viral genome in cDNA or RNA to understand and study viral genetics (Perez, 2017). These powerful platforms are used for modifying the viral genome to study the replication and pathogenesis of multiple viruses. This system also allowed the successful genetic modification of RNA viruses and the production of recombinant RNA viruses for vaccination or different therapeutic purposes (Peeters and De Leeuw, 2017). These recombinant viruses especially with picornaviruses have shown their potential in cancer therapy development due to their selective infection, cell lysis ability, and receptor binding affinity. One example is the rescued modified coxsackievirus group B (CVBs) virus express luciferase, preclinical studies have shown their anti-tumor activities against multiple cancers (Liu and Luo, 2021) . This method is very effective for virus rescue experiments. Scientists first convert the viral RNA into complementary DNA for manipulation and then introduce these cDNA clones into suitable host cells. The viral cDNA is then transcribed into functional viral RNA using the host cell's machinery and ultimately generates infectious virus particles (Wang et al., 2024). In another way, virus rescue means, when full-length viral cDNA clones are transfected into a compatible host cell, the reconstructed viral genome is transcribed and translated using the cell

machinery after successful infection, and new infectious virus particles are produced from that modified virus (Li et al., 2021). Virus rescue experiments can be done in several ways, one is using *in vitro* technique. The viral RNA is converted into cDNA in a small Eppendorf tube and then transfected to the host cells for viral replication. Plasmid-based virus rescue methods start by cloning the full-length viral genome into a plasmid vector containing the essential regulatory elements like strong promoters (e.g. T7, CMV, or SV40), and after transfecting into host cells the promoter drives the transcription of viral RNA using host cell's machinery for assembly and release viral particles (Stobart and Moore, 2014). Racaniello and Baltimore used this plasmid-based approach with poliovirus in 1981 (Racaniello and Baltimore, 1981). Another way is using different strong promoter-based PCR techniques for rapidly rescuing viruses. This process usually starts with using reverse transcription for converting viral RNA into cDNA, and then specific primers containing different powerful promoter sequences based on the system such as T7, CMV, or SV40 promoters are used for the full-length amplification of the viral genome. The PCR amplicons then transfect into the host cells to generate infectious viral particles (Heikkilä et al., 2011). However, there are some challenges in reverse genetics, such as identifying the function of specific genes due to the complex genomic structure, the delivery methods need to be optimized to deliver the genome into host cells, and finally interpreting the results is difficult because of the complexity of gene interactions. Challenges also depend on virus type and strain, and the compatibility of the cell lines used for viral propagation.

1.9 Motivation of the investigation

From the 7th-century smallpox to last year's coronavirus pandemic, viruses are the main reason behind some major outbreaks and are responsible for the death of millions of people. In the past 100 years, investigating pathogenic microorganisms has advanced significantly (Enquist, 2009). Studying viruses is challenging as viruses lack their metabolic machinery and completely depend on host cells for replication (Thaker et al., 2019). Thereupon, it is necessary to establish straightforward, and reliable protocols for efficient virus research, which allows researchers to try new strategies to accelerate the development of new antiviral drugs and vaccines, gene therapy, targeted therapy for cancer cells, and many more (Carroll et al., 2021). Although there has been a significant advancement in the virology field, such as the development of antiviral drugs, viral vaccines, and immunostimulators for killing virus-infected cells, there is still a huge scientific knowledge gap, lack of proper virus cloning methods, novel vaccines, antiviral drugs screening or effective antiviral treatment methods. Establishing new

methods or improving the existing protocols in virology, helps researchers to plan and organize experiments much simpler and more straightforwardly.

Picornaviruses are responsible for several human and animal diseases, conditions ranging from mild respiratory infections to severe illness. We don't have any direct antiviral drugs against these viruses and barely have vaccines against these viruses. Although these viruses cause devastating diseases, modified Picornaviridae family viruses have shown promising cancer therapy due to their ability to bind specific cell surface receptors and lyse cancer cells. Using these viruses to develop as vectors and then incorporating marker genes into their genome can help us understand viral behaviours.

This study aims to modify the coxsackievirus A9 (CVA9) virus by incorporating marker genes such as the green fluorescence protein (GFP) gene to understand the virus's properties (Fields, 2013). The T7 RNA polymerase-based systems can be used for rescuing modified virus particles (Racaniello, 2006). Strong promoters such as T7-promoter are incorporated into the CVA9-eGFP viral genome, which drives the viral transcription by utilizing these T7 RNA polymerase enzymes synthesized from the cells. In some virus vector studies, viruses are modified so that they contain markers like fluoresce encoding genes which help visualize the virus. In antiviral drug screening studies, cells can be treated previously with newly developed antiviral drugs, and virus vectors containing marker genes transfect into those cells. After infection, if there is any fluorescence activity observed, indicates that the virus replicates within the cells even though cells were treated with antiviral drugs previously. This kind of screening experiment helps us to assess the effectiveness of newly developed antiviral drugs (De Clercq, 2002). Several factors motivated this study, such as using the CVA9-eGFP vectors for the delivery of therapeutic genes, and oncolytic agents into targeted cells, establishing a helper virus-free viral transcription system, optimizing long PCR methods for robust amplification, and transfection conditions. Detailed analysis of the CVA9-eGFP viral vector can be used for assessing transfection efficiency, tracking viral infection and replication, and evaluating the stability and expression of the inserted genes (Chalfie et al., 1994). For any kind of virus research, plasmid cloning is very important for conducting research, as viral genomes are inserted into those plasmids. Therefore, it is necessary to have a simple, straightforward, and effective method for generating cDNA versions of viral genomes (Russell et al., 2012). This study also aims to improve our understanding potentiality of CVA9-eGFP viral vectors for gene delivery and cancer therapy.

1.10 Research questions & hypothesis

The fundamental objective of this thesis is to investigate the functionality and behaviour of a genetically modified picornavirus vector, i.e., the coxsackievirus A9-eGFP vector bearing a green fluorescent protein (eGFP) encoding marker gene, within mammalian cell cultures. The primary questions of this research are whether the CVA9-eGFP vector can function properly within mammalian cell cultures and whether it can be used in studies of mutagenesis, virus tropism, and pathogenesis, and for developing cancer therapies. Besides, this research also tries to find out if the T7 RNA polymerase (T7RNAP) system can be used for in vivo rescue of CVA9 and CVA9-eGFP virus particles from viral cDNA or T7-promoter-tagged genomic amplicons, along with if the full-length 7.5 kb CVA9 genome can be robustly amplified by using an optimized long-PCR protocol. This project also aims to explore the efficiency of several promoters, i.e., the T7 promoter and the CMV promoter, for the rescue and replication of the Picorna viral genome. Identifying the most efficient and effective cell transfection method for delivering the viral genome into mammalian cells for obtaining the highest transfection efficiency and successful in vivo virus rescue is also a key concern of this project. This project wants to test whether the CVA9-eGFP vector can be accurately tracked by using fluorescence microscopy to monitor viral infection and replication in real-time (Chalfie et al., 1994; Heikkilä et al., 2011).

The main hypothesis driving this project is that the CVA9-eGFP vector can function properly within mammalian cell cultures, which allows real-time tracking of viral infection and replication via fluorescence microscopy. This project also hypothesized that the T7RNA polymerase system can successfully rescue functional infectious CVA9 virus particles from direct cDNA clones or T7-promoter-tagged PCR amplicons in mammalian cells (Studier, 1991). The full-length CVA9 viral genome can be robustly amplified by incorporating a T7 promoter at the 5´-end of the viral genome that can drive effective viral RNA transcription and subsequent virus rescue within mammalian cells, which is another hypothesis of this study (Heikkilä et al., 2011). For effective viral RNA transcription, it is expected that the T7 promoter will need T7 RNA polymerase (T7RNAP) for virus rescue, while the CMV promoter-based transcription doesn't need the T7RNAP system. Another hypothesis for this study is that the co-transfection of CVA9 and CVA9-eGFP plasmids with the CMV promoter-driven T7 RNA polymerase-encoding plasmids in both BHK-21 and A549 cells can generate infectious virus particles. Moreover, this project hypothesizes that optimized transfection conditions such as DNA and transfection agent concentrations, ratios, cell confluency, etc. will significantly

improve CVA9 viral genome delivery into mammalian cells and virus rescue (Heikkilä et al., 2011).

By investigating these research questions and testing these hypotheses, we want to provide valuable information about the CVA9-eGFP vector, which can be used for developing gene therapy and targeted therapy and also advancing our knowledge in the field of molecular virology research. To develop noble therapeutic agents for treating viral diseases, this project aims to establish an efficient method for rescuing viruses and successful genome amplification by PCR techniques (De Clercq, 2002).

1.11 Experimental framework

There were several steps taken to accomplish this research experiment. The first goal of the project was to isolate and purify different clones of coxsackievirus A9 with eGFP(s) (including CVA9-eGFP6, CVA9-eGFP7, and CVA9-eGFP11) genome containing plasmids, as well as pCMVT7RNAP & pCMV/T7-T7RNAP having T7 polymerase encoding gene insert. CVA9-eGFP is a modified form of CVA9, which contains an eGFP gene (700bp) inserted into the genome that can produce eGFP protein, i.e., green fluorescence protein. When enough amount of plasmid DNA is purified from the transformed bacterial cell culture using purification kits, the plasmid construct is verified through 1% agarose gel electrophoresis. The second goal was to create T7-promoter-tagged PCR amplicons so that these PCR amplicons utilize T7 polymerase enzymes directly in T7-BSR (BHK hamster cell line constitutively expressing T7 RNA polymerase enzyme, (Buchholz et al., 1999)) cells and generate virus particles. The long-PCR protocol uses CVA9-eGFP as a template with the most optimal PCR enzymes obtained from previous studies (Koskinen, 2023), which was implemented in this experiment to amplify the whole viral genome. The long-PCR primers were designed in such a way that they contain the T7 promoter and are complementary to the target sequence at the 5´- end of the CAV9-eGFP genome. Several reverse primers were used to compare the replication efficiency to understand the effect of the primer sequence in virus replication. The PCR products were analyzed on agarose gel and imaged under UV light to verify the success of the PCR reaction, after the analysis the remaining PCR products were purified by purification kits according to the manufacturer's manual. In the third stage, transfecting mammalian cells using appropriate transfection agent with all the T7-PCR amplicons, and direct pCVA9, pCVA9-eGFP (s) plasmids. The T7 approach required T7-BKH cell lines particularly as these cells directly produce the T7 polymerase enzyme, which is crucial for virus replication (Buchholz et al.,

1999). This method eliminates the use of any helper virus to deliver T7 polymerase enzyme into the cells. 2/3 days later, infected cells were transferred to a new fresh medium with end-point titration, to obtain virus particles. Finally, the CVA9-eGFP infected cells were observed and imaged using an EVOS FL AUTO fluorescence microscope. The eGFP gene produces a green fluorescent protein, which allows us to detect the virus in the cell without separate antibody staining.

2 Materials and methods

2.1 Plasmids and cells

In this experiment, a total of six plasmid DNAs were used, comprising four different forms of pCVA9 and two plasmids bearing the T7 polymerase enzyme encoding genes. pCVA9 (coxsackievirus A9, Griggs strain, GenBank acc. no D00627, 7500 bp), and 3 different pCVA9-eGFP forms as pCVA9-eGFP6, pCVA9-eGFP7, and pCVA9-eGFP11 (green fluorescent protein-encoding gene cloned into VP1-2A junction of CVA9 cDNA backbone plasmid, 8200 bp, eGFP 700bp, (Heikkilä et al., 2011)) were from the Picornavirus laboratory collection. These three different forms with the GFP gene are different plasmid clones and they contain ampicillin resistance selection markers. CMV promoter-based plasmid, pCMVT7RNAP (containing cytomegalovirus promoter, 10297 bp, (Meyer et al., 2004)) was collected from Dr. Ralph Meyer (University of Tübingen, Germany), and pCMV/T7-T7RNAP (constructed by inserting HINDIII/BamHI fragment from Par3126 into corresponding sites of the pcDNA3 plasmid vector, 8400 bp, (Brisson et al., 1999)) was from Dr. Karl-Klaus Conzelmann (Federal Research Center for Virus Diseases of Animals, Tübingen, Germany). There were two different cell lines used for cell transfection, are BHK-21 cells (baby hamster kidney cells) and T7-BSR (BHK-21 derivative, (Buchholz et al., 1999)). This BHK-21 cell line was a gift from Conzelmann, while T7-BSR cells were from Dr Brisson.

2.2 Cultivation of bacteria

Pre-mixed powder of LB media containing Tryptone, NaCl, and Yeast Extract all the necessary growth nutrients crucial for bacterial growth. To make 1000 mL of LB media, 950 mL of MilliQ H2O was mixed with 25 gm of pre-mixed LB powder until the powder was dissolved. Before using or storing the media it was autoclaved. For making 50 mL LB agar (for 3 plates), 50 mL deionized water with ready-made LB powder, and 0.75g agar powder are mixed until fully dissolved and autoclaved before pouring the agar media into plates. Antibiotics were added when the media cooled down to around ~50-55°C.

2.3 Heat-shock transformation of bacteria

Commercially available competent Escherichia coli (One Shot TOP10, Invitrogen Inc.) bacterial strain similar to genotype DH10B (genotype: F–mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG

λ–) cells stored at -80°C, were thawed on ice for few minutes. 5 µL of each plasmid DNA sample were directly pipetted over 20-40 µL of competent E. coli cells, mixed by gently tapping, pipetting up and down avoided, and incubated on ice for 30 minutes. Placed the tubes in the water bath to apply heat-shock at 42°C for 45 seconds, and then incubated on ice for 2 minutes. 0.5 mL of LB medium was added to each Eppendorf tube and after that, the transformed cells were immediately spread on LBA plates using a plastic spatula. 50 mL of LB agar (for 3 plates) made by 0.5 gm bacto tryptone, 0.25 gm yeast extract, 0.5 gm NaCl, and 0.75 gm of bacto agar. Each plate contained antibiotics according to the plasmid DNA, such as pCAV9 (AmpR, 100 µg/mL), pCAV9-eGFP (AmpR), pCMV/T7-T7RNAP (AmpR), and pCMV-T7RNAP (KmR 50 µg/mL) used in for the transformation. Plates were inverted and incubated at +37°C overnight.

2.4 Colony picking

To cultivate the transformed bacteria, a single colony was picked from each plate using a pipette tip and transferred in 5 mL snap-cap Falcon tubes having LB medium with appropriate antibiotics (AmpR, 100 µg/mL or KmR 50 µg/mL). These Falcon tubes were then incubated at 37°C with vigorous shaking at 225 rpm overnight.

After the overnight cultivation, 1 mL of those bacterial cultures were transferred in storage Eppendorf tubes spun down to the pellet, and the supernatants were removed. Later, the pellet was suspended with 40% glycerol-LB medium and stored at -80°C for future use. The remaining 4 mL was spanned down to generate the pellet. Supernatants were discarded and stored dry at -80°C for using later plasmid purification experiments.

2.5 Plasmid DNA extraction and purification

Isolation of plasmid DNA from transformed Escherichia coli strains is crucial for this work. Easy-to-extract proprietary kits in different formats such as mini, midi, and maxi column-based protocols were used for this experiment. The optimized methods for extracting plasmid DNA based on the quality and quantity of plasmids are looking for. Several commercial kits were used to isolate and purify high-quality, endotoxin-free DNA from bacterial culture. To purify a small amount of Plasmid cDNA from less than 10mL of bacterial cell culture commercially available NucleoSpin Plasmid Transfection-grade Mini prep kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's instruction on user manuals. 5 mL of overnight culture was centrifuged, the supernatants were suspended with a user-recommended

buffer, and the plasmid as per company instruction. After the mini prep, the extracted DNA was diluted in 50 µL NucleoSpin elution buffer, and the concentration was measured on the DeNovix DS-11 nanodrop spectrophotometer (DeNovix Inc., Wilmington, USA). For generating long-PCR amplicons and cell transfection experiments, the larger volume of high-quality plasmid DNA was collected by using commercial maxi prep kits. GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, Darmstadt, Germany) uses a vacuum format filter column for isolating plasmid DNA. For this, 2 mL of pre-cultured bacterial cells were transferred to a larger 500 mL LB medium and cultivated overnight (+37oC, 225 rpm). After centrifuging the bacterial culture, the purification started as per the manufacturer's protocol. The purified plasmids were eluted in 12.5 mL of elution buffer and the concentration was measured using the DeNovix DS-11.

2.6 Restriction mapping and digestion of plasmids

The purified plasmids concentration was measured by DeNovix DS-11 nanodrop spectrophotometer (DeNovix Inc., Wilmington, USA) using the manufacturer's user manuals. Both pCAV9 (GenBank acc. no D00627) and pCAV9-eGFP (Heikkilä et al., 2011) plasmids sequences were analyzed for establishing restriction map by using the NEBcutter (https://nc3.neb.com/NEBcutter/) program from New England Biolabs and restriction enzymes were selected. FastDigest restriction enzyme ClaI with Tango Buffer (yellow buffer) (65°C, 60 minutes for heat inactivation, Thermo Fisher Scientific) was used for digest pCAV9, while pCAV9-eGFP (s) were digested by FastDigest restriction NotI (80°C, 5 min heat inactivation, Thermo Fisher Scientific) enzyme. For pCMV-T7RNAP (Meyer et al., 2004) (10200 bp) FastDigest restriction enzymes including Hind III, EcoRI (80oC, 5 min heat inactivation, Thermo Fisher Scientific) were used. In pCMV-T7/T7RNAP (Brisson et al., 1999) (8400 bp) enzymatic digestion, Hind III (65°C, 5 minutes for heat inactivation, Thermo Fisher Scientific), EcoRI, and HIND III+ BamHI (80°C, 5 min heat inactivation, Thermo Fisher Scientific) enzymes were used. All the FastDigest restriction enzymes were inactivated by thermal inactivation according to the manufacturer's user guide on the website.

For restriction mapping analysis, 2 µL of purified DNA solution (0.2 µg/µL) was incubated at 37°C for at least 2 hours, with specific restriction enzymes. The reaction mixture had a final volume of 20 µL, bearing restriction buffer and water, prepared according to the manufacturer's instructions, and the reaction mixing chart is presented in Table 1.

Table 1 Typical Restriction enzyme pipetting chart (for one sample): calculate master n + ½ or 1 (n = number of samples)

Reagent	**Volume (µL)**
10x buffer	2
ddH2O	X
Plasmid	Y
R-enzyme	0.5
Total	**20**

All the digested plasmids were analyzed on a 0.8-1.5% agarose gel (Metaphor agarose; FMC, BioProducts, Risingevej, Denmark) prepared in 1x Tris-EDTA (TAE) buffer with 0.5 µg/mL ethidium bromide.

2.7 Amplification of CVA9 and CVA9-eGFP genome by PCR

A full-length CVA9 genome was amplified from pCVA9 using a 5'-end primer (pCAV9gen1_T7_F) that contains T7-promoter (T7p) region (Hughes et al., 1995) with three G residues and complementary region for the CVA9 immediately after three G´s and three different regulatory 3'-end primer sets (pCVA9gen1_R, CAV9gen1-R_T7-T^{orig}, CAV9gen1-R_T7-T^{synt}) producing a stable poly-A-tail of 20 bases. The PCR reaction was performed using high-fidelity Platinum SuperFi II DNA polymerase enzyme (Thermo Fisher Scientific, CAT# 12361010). The cycling conditions for this PCR run were a 60 s initial denaturation at 98°C, 30-40 cycles with 15 s for complete denaturation at 98°C, then 20 s for annealing at 67°C, followed by 4 min extension at 72°C. The final extension at 72°C for 7 min 30 s and at 4°C the reaction held (Koskinen, 2023). All PCR reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems™ by Thermo Fisher Scientific). The method used for preparing the PCR reaction mixture is presented in Table 2.

Table 2 Reaction mixes for PCR enzyme used.

Reagent	**Volume (µL)**
Nuclease-free water (ddH_2O)	12.2
5 x PCR buffer	4
dNTP mix (10 mM)	0.4 (200 µM)
F- primer (10 µM)	1 (0.5 µM)
R-primer (10 µM)	1 (0.5 µM)
Template DNA (10 ng)	1
Platinum SuperFi II	0.4
Total volume	**20µL**

Three separate sets of reverse primers (pCVA9gen1 T7 F, CAV9gen1-R_T7-T^{orig}, and CAV9gen1-R_T7-T^{synt}) with one common forward primer (pCVA9gen1_T7_F) were used to amplify the full-length CVA9 and CVA9-eGFP (s) genomes through long-PCR techniques to generate T7-promoter-tagged PCR amplicons. Table 3 presents the detailed information about the primer sets.

Table 3 Primers used for PCR. Complementary section underline. F= forward R= reverse

Primer	**Sequence**	**Phusion Tm**
pCVA9gen1_T7_F	5'-TAATACGACTCACTATA GGGTTTAAAACAGCCTGTGGGTTGTTCCC-3'	71,8°C
pCVA9gen1_R	5'-TTTTTTTTTTTTTTTTTTTT CCTCCGCACCGAATGCGG-3'	74,7°C
CAV9gen1-R_T7-T^{orig}	5´-AACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCU-3´	83.7°C
CAV9gen1-R_T7-T^{synt}	5´-AACCCCGCGGGGCCTCTTCGGGGGTCTCGCGGGGTTTTTTGCT-3´	87.0°C

2.8 Purification of PCR product

The PCR products were analyzed on gel electrophoresis after the reaction was complete. 2 µL of each PCR product was run on 1% agarose gel for 1 hour at 100V. The remaining amounts were purified by using the NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL, Düren, Germany) commercial kit, following the manufacturer's manual guide. Clean PCR products were diluted in 25 mL of elution buffer NE, concentrations were measured using the DeNovix DS-11 nanodrop spectrophotometer and stored at -20°C.

2.9 Agarose gel electrophoresis

The restriction enzyme-digested products and PCR products were analyzed on the agarose gel. Weight 2 gm of agarose powder (1% gel) mixed in 200 mL of 1x TAE buffer in a 450 mL Erlenmeyer flask. Microwave for 2 minutes for melting, cool down to ~50°C, then add 12 µL of Midori Green Advance DNA Stain dye (Nippon Genetics, Düren, Germany) (0.5 µg/mL), and cast the gel mixture in 15 well gel combs. 2 µL of sample diluted in loading buffer (6X TriTrack DNA Loading Dye, Thermo Fisher Scientific) were loaded in each well separately, and the gel ran on a 100V electric field until separate bands had formed.

GeneRuler DNA Ladder Mix (Highest 10000 bp, Thermo Fisher Scientific) and GeneRuler 100 bp Plus DNA Ladder (Highest 3000 bp, Thermo Fisher Scientific, Pub. No. MAN0013008) were also loaded in the first and last well of the gel. The Bio-Rad Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories, Inc.) was used for taking the gel image, and then Image Lab (Bio-Rad Laboratories, Inc.) software was used for gel image analysis.

2.10 Mammalian cell cultivation and transfection

The plasmid forms of CVA9, CVA9-eGFP (s), and their T7-promoter-tagged PCR amplicons were transfected into two mammalian cells. BHK-21 and T7-BSR cells were propagated in Dulbecco’s minimal essential medium (DMEM medium, BioWhittaker, 12-709F) with 10% fetal bovine serum (FBS, GIBCO Life Technologies, USA) and maintained at standard cell culture conditions with +37°C in a humidified atmosphere containing 5% CO2 in T-75 flasks. The T7-BSR cells were mainly used for virus production due to their ability to T7 polymerase enzyme expression, while the original BHK-21 cells were used as a control. The cell cultures were diluted and passaged every 3/4 days. Cells were detached from the bottle with 1X Trypsin-EDTA, diluted to PBS, and cell viability and number were measured by trypan blue exclusion

using TC20TM Automated Cell Counter (BIO-RAD). According to the manufacturer's guidebook, these cells were routinely transfected using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, L3000075). T7-BSR cells were seeded in black 96-well (Perkin Elmer plates, 6005050) plates were pipetted with approximately 20,000 cells/well, which were grown overnight at +37°C and 5% CO2 approximately 70-90% confluence in DMEM medium with 10% fetal bovine serum (FBS), 10 µg/mL gentamicin antibiotic (Biological Industries, 03-035-1B) and 200 µg/mL Gibco Geneticin antibiotic (Thermo Fisher, 10131035). For BHK-21 cells, 20,000 cells/well were grown overnight at +37°C and 5% CO2 approximately 70-90% confluence in DMEM medium containing 10% FBS serum in a 96-well plate, and the medium was changed after 24 h. All the plasmids and T7-PCR amplicons were transfected into the cells and two parallel wells of a well plate (Perkin Elmer Black plates) and 24 h later the medium changed. In infection, a DNA samples mixture was formed according to the manufacturer's user manual instructions of the Lipofectamine™ 3000 Reagent, using a sample volume of 2.5 µL /well (1:1), containing 0.15µL/well (1:1) Lipofectamine™ 3000 Reagent, which diluted in serum-free DMEM medium. Three different plasmid/PCR product concentrations versus transfection agents (3:1, 1:1, and 1:3) were used. The cells' culture medium was changed to 100 µL of serum-free DMEM medium before pipetting the cell sample mixture, and the total final volume was 110 µL. pGFP (plasmid with GFP) was used as a transfection control in all different versions of transfection. End-point titration was carried out in black plates with 10000 cells/well. 100 µL medium was changed on the following day, and 10 µL of each sample was added to the first row, then serially diluted the sample down to the 4th row, now discarding the 10 µL after mixing, producing 4 wells with 10-fold dilutions of the virus, using a multichannel pipette. After 48 hours, cells were fixed in 4% (para) formaldehyde (PFA) for 10 min. Cells were rinsed twice with 100 µL Phosphate-buffered saline (PBS), and 60 µL of DAPI (4′, 6-diamidino-2-phenylindole, Thermo Fisher Scientific) diluted 1:2000 ratio in 3% BSA (bovine serum albumin) solution in PBS was added to each well and incubated in the dark for 10 min. Cells were then washed two times with PBS for 30 seconds each wash. Cells were imaged with an EVOS FL AUTO fluorescence microscope (Invitrogen, Thermo Fisher, and AMAFD1000). Wells containing cells were imaged in a way, cell nuclei were imagined in the blue (DAPI mood) light channel, and cells transfected with CVA9-eGFP were imaged in the green channel as green fluorescence visible under GFP (green) mood.

3 Results

3.1 Plasmid purification and verification

All plasmids used for this project originated from past projects, and in this part, I needed to verify which one of those clones was useful for my experiments. I had one original CVA9 clone and three 3 different CVA9 clones with GFP which were CVA9-eGFP6, CVA9-eGFP7, and CVA9-eGFP11 and I wanted to test if they were functional after virus rescue. Then I have different T7 including T7RNA polymerase encoding plasmids pCMVT7RNAP and pCMV/T7-T7RNAP which I also wanted to test what is their functionality in cotransfection. All the plasmids were transformed into bacteria (E. coli) through heat-shock transformation, and incubated overnight. An overnight culture was prepared with each plasmid by inoculating a sterile growth medium with a single bacterial colony picked from the agar plates and followed by overnight incubation. Plasmids were purified from those bacterial overnight cultures by using commercial miniprep kits in small amounts.

I used restriction enzyme analysis to verify that any of those clones used for this project were okay and to confirm the presence of the gene of interest, and those plasmid clones were then selected for the next stage. Some clones were stopped using because their plasmid construction was not fine. FastDigest restriction enzymes, selected based on the plasmid's genomic structure, were used to digest the plasmid for restriction analysis. Single-cutter FastDigest enzyme ClaI with tango buffer was used to digest the original CVA9 plasmid, which cut the plasmid in a specific position to open up the 7.5 kb circular plasmid. On agarose gel analysis (Figure 4) this CVA9 digested plasmid produced a band between 8 kb and 6 kb ladder mix indicating that the plasmid contained the CVA9 genome. The other 3 CVA9 with GFP (s) total length of 8.2 kb were digested using double cutter FastDigest NotI which was cut in two positions to separate the GFP gene from the backbone CVA9 plasmid vector. This enzyme digestion generates two bands on agarose gel, the CVA9 backbone at 7.5 Kb and the GFP band visible between 700 bp to 800 bp (Figure 4). 5 µL of plasmids were digested by 0.5 µL of enzymes so that all the plasmids used during this reaction was not fully digested during the incubation and showed some undigested bands (Figure 4) on agarose gel.

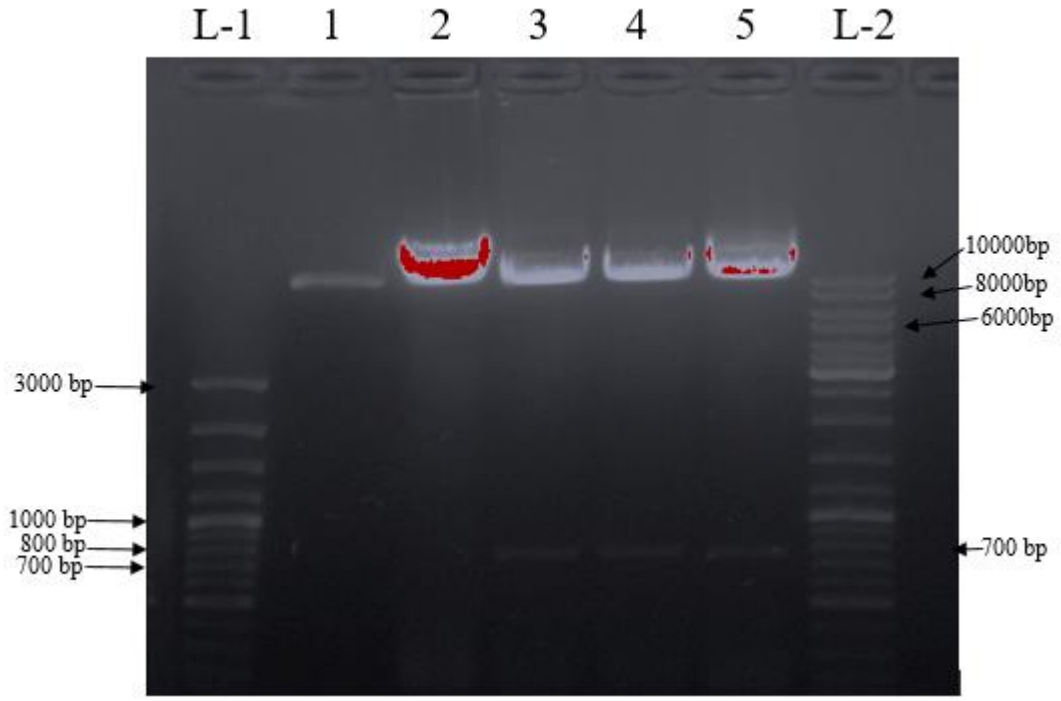

Figure 4 Gel analysis of restriction enzyme digested pCVA9 (7500 bp) and pCVA9-eGFP (s) (8200 bp). L-1: Marker (GeneRuler 100 bp DNA ladder); lane- 1: CVA9 digested by ClaI: lane 3- CVA9-eGFP6; lane 4: CVA9-eGFP7; lane 5: CVA9-eGFP11 all three were digested by NotI; L-2: Marker (GeneRuler 100 bp DNA ladder).

As mentioned above, several T7 RNA polymerase-encoding plasmids were used from previous work. 10 different pCMV-T7RNAP clones, each with a total length of 10,297 bp, were isolated, purified, and analyzed by restriction digestion. All the pCMV-T7RNAP clones were analyzed using two restriction enzymes, such as Hind III and FastDigest KpnI enzyme. FastDigest Hind III is a single cutter that linearized the T7RNAP circular plasmids and FastDigest KpnI is a double cutter that produced two separate fragments 2.5 kb and 7.7 kb. On agarose gel analysis (Figure 5) plasmids digested by Hind III showed a single band slightly above the 10 kb DNA ladder marker, and KpnI-digested plasmids on the gel (Figure 5) produced two bands one was between 8 kb and 7 kb, another nearly 2.5 kb marker ladder. Not all the clones showed intense bands on agarose gels (Figure 5). A total of seven clones that were successfully digested by restriction enzymes (lanes 1, 2, 3, 5, 6, 7, and 10) were considered okay and among them, only one was selected for the next experiments and unsuccessful clones (lanes 4, 8, and 9) were excluded from this project. Among these plasmids, only one clone (lane 1) was used for the next experiments, and other plasmids were stocked for future use.

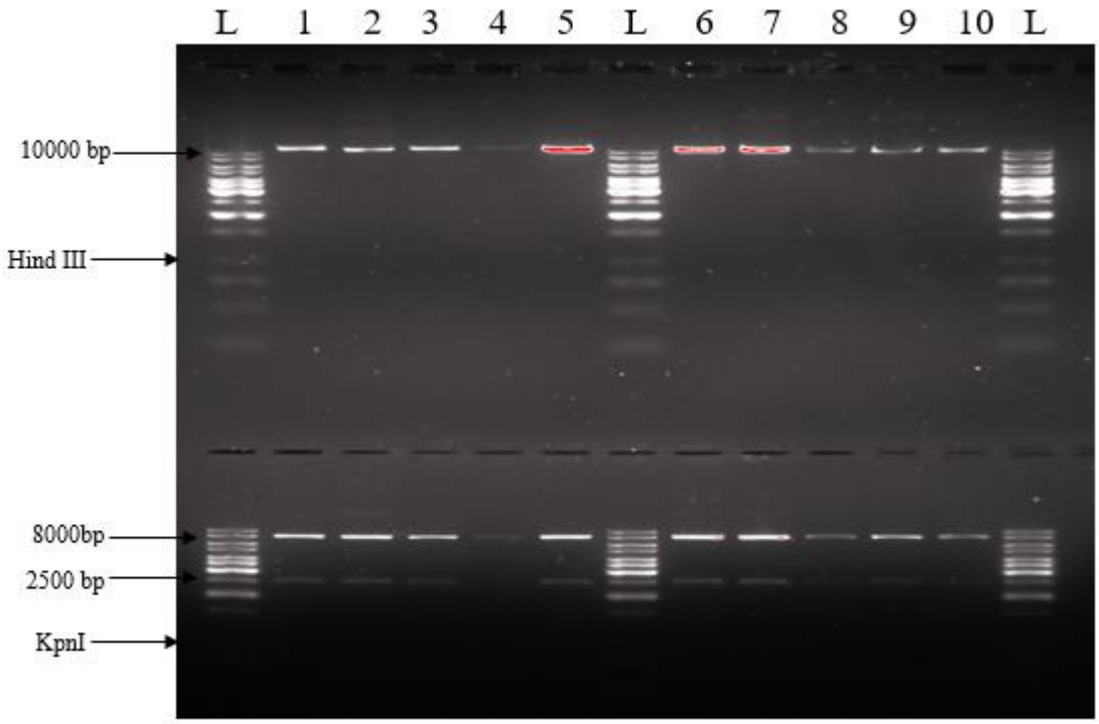

Figure 5 Analysis of restriction enzyme digestion reactions of pCMV-T7RNAP (10,297 bp). In the first row samples were digested by Hind III, and in the second row by KpnI restriction enzyme. Lane L: Marker (1 Kb GeneRuler DNA ladder); Lane 1-5 and Lane 6- 10: different pCMV-T7RNAP clones analysis by restriction digestion.

One pCMV-T7/T7RNAP plasmid containing the T7 RNA polymerase encoding enzyme gene was purified and verified by restriction analysis. CMV-T7/T7RNAP plasmid clone was digested by EcoRI, Hind III, Hind III, and BamHI restriction enzymes, and run on agarose gel for analysis. There was some issue with the EcoRI enzyme digestion as they showed several bands on the gel. The Hind III is a single cutter that linearized the 8400 bp circular CMV-T7/T7RNAP plasmid and produced a single band on lane 2 (Figure 6) of agarose gel. The Hind III enzyme digested plasmids accumulated slightly above the 8 kb band compared to the standard ladder mix, which verified this plasmid clone containing the T7 RNAP gene was okay. Enzymatic digestion with both Hind III and BamHI verified the plasmid construct as these two enzymes produce two fragments of 3 & 5.4 Kb through double digest. On agarose gel (Figure 6) one band was nearly 6 Kb, and another was slightly up the 3 kb standard ladder which was expected according to the restriction mapping. This restriction digestion verified that the CMV-T7/T7RNAP plasmid was okay for future vector analysis.

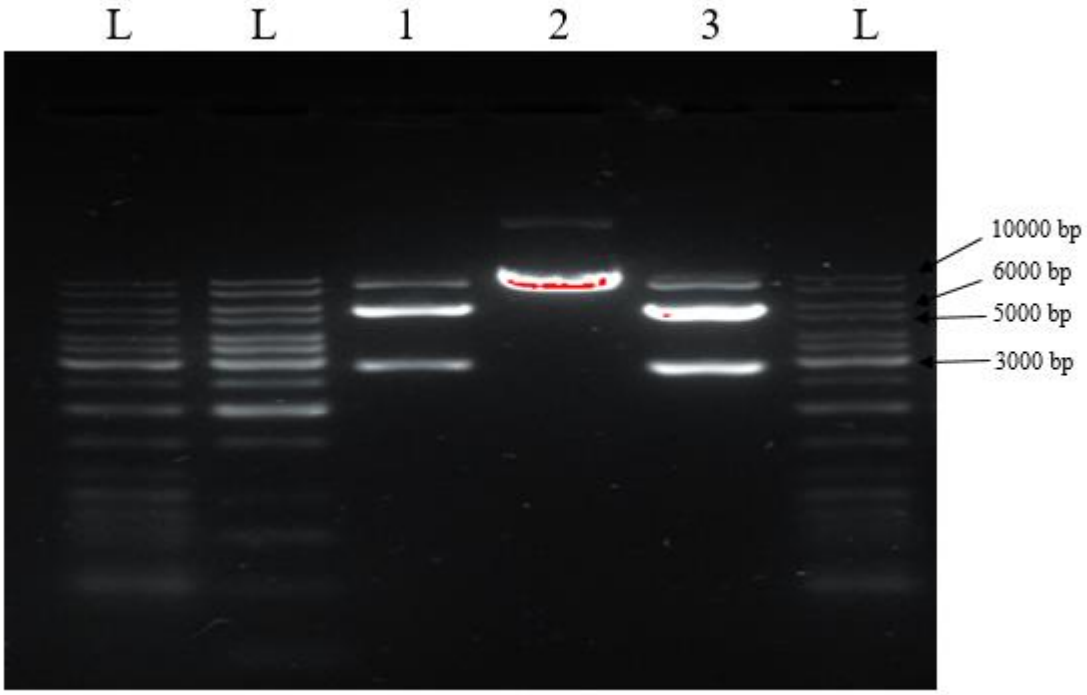

Figure 6 Agarose gel electrophoresis of restriction digestion analysis of pCMV-T7/T7RNAP (8,400 bp) by EcoRI, Hind III, and Hind III+ BamHI. Lane L: 1 Kb GeneRuler DNA ladder; lane 1, plasmid digested with EcoRI; lane 2, plasmid digested with Hind III; lane 3 plasmid digested with Hind III + BamHI.

When all the plasmids construct were verified by restriction enzyme analysis, the selected six plasmid clones, such as pCVA9, pCVA9-eGFP6, pCVA9-eGFP7, pCVA9-eGFP11, pCMV-T7RNAP (T7 RNA polymerase encoding), and CMV-T7/T7RNAP were cultured in larger volume (400 mL) for purifying 10 mL plasmid DNA using commercial maxi prep kits. The concentration of those plasmid clones was measured and these concentrations were used for further experiments. The concentration is presented in Table 4.

Table 4 Concentration of plasmids.

Plasmid Name	Concentration (ng/ µL)
pCVA9	341.527
pCVA9-eGFP6	654.007
pCVA9-eGFP7	497.239
pCVA9-eGFP11	176.869
CMV-T7RNAP	124.493
CMV-T7/T7RNAP	132.007

3.2 Generation of T7-promoter-tagged CVA9 and CVA9-eGFP PCR amplicons

To rescue CVA9 virus particles using the T7 system, T7-promoter-tagged PCR amplicons were developed. T7-PCR products were prepared with long-PCR, where specific primer sets were used to produce PCR products which are in the form of cDNA spanning the full length of the entire viral genome. In addition, novel primers set (Table 3) were tested during amplification to generate FL-CVA9 PCR amplicon using the high-fidelity Platinum SuperFi II DNA polymerase enzyme. The T7 promoter region is designed as a forward primer named pCVA9gen1_T7_F in front of the 5' end of the genome (Hughes et al., 1995). Other three different regulatory reverse primer sets were used (Table 3) to test their role in viral replication. T7 PCR products were successfully produced by a two-step PCR reaction (Figure 7) with pCVA9 and other 3 forms of CVA9 with the GFP gene including pCVA9-eGFP6, pCVA9-eGFP7, and pCVA9-eGFP11 were used as a template. The other reverse primers were used to generate a long poly-A tail to stabilize the transcription. The T7-PCR amplicons of all pCVA9 and pCVA9-eGFP (s) were generated using the protocol mentioned in the 2.7 section. The plasmids used as the templates for the PCR reaction were set to the concentration of 10 ng/ µL to ensure consistency and minimize the variation in templates in the reaction. In the first PCR reaction, 20 µL of amplified T7-tagged PCR amplicon was generated from 1 µL of DNA template. The amplified PCR fragments concentration was measured by spectrophotometer.

The success of the PCR reaction was then analyzed through agarose gel electrophoresis, which separates the DNA products based on size (Figure 7). 5 µL of PCR amplicons were mixed with 1 µL of 6X TriTrack DNA loading dye (Thermo Fisher Scientific) and loaded the mixture into the agarose gel. The CVA9-T7-PCR amplicon showed a band (Figure 7) in between the 6 kb and 8 kb band of the ladder, which verify the amplification of the genome was successful as the total size of the CVA9 genome was 7.5 kb. The CVA9-eGFP (s) produced bands on gel slightly

above 8 kb of the ladder, as the total length of the CVA9-eGFP genome was 8.4 kb which also verified the PCR amplification of the genome was successful.

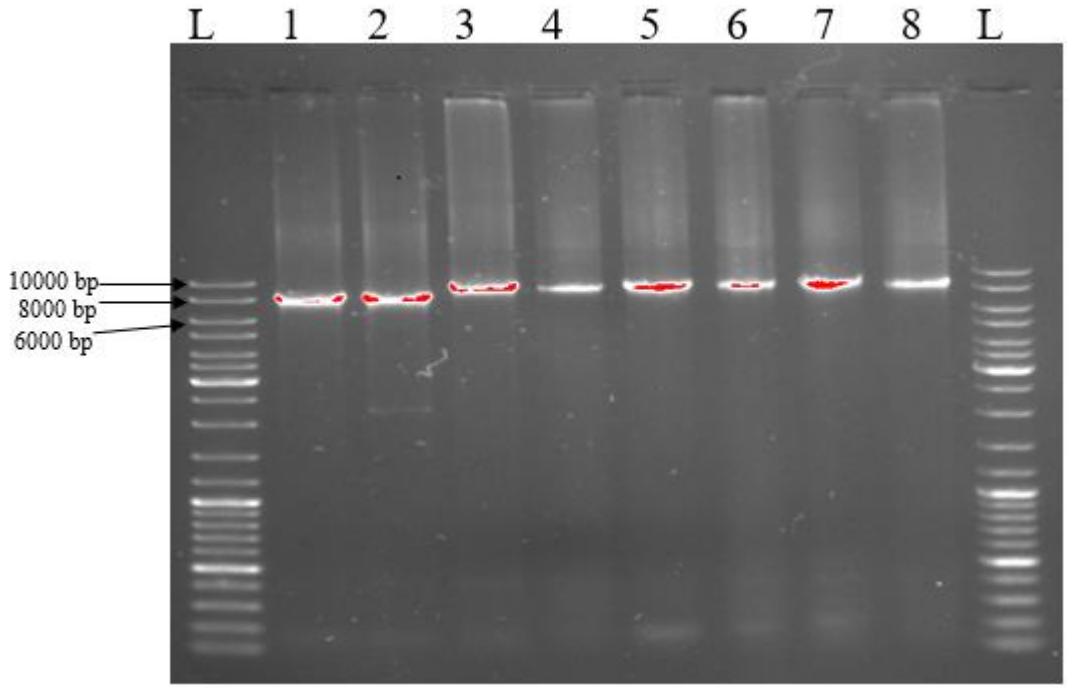

Figure 7 Analysis of the CVA9 PCR amplicon in 1% agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide. Amplification of full-length CVA9 and CVA9-eGFP genome by T7-promoter containing primers. Lane L: GeneRuler 1 Kb DNA ladder mix (Marker starts from 10 Kb). Lanes 1 & 2 CVA9 (nearly 7.5 bp), Lanes 3 & 4 CVA9-eGFP6 (around 8.4 kb), Lanes 5 & 6 CVA9-eGFP7 (~ 8.4 kb), and Lanes 7 & 8 CVA9-eGFP11 (8.4 kb).

3.3 Transfection of T7-CVA9 to BHK and T7-BSR cells

We tried to rescue the infectious coxsackievirus A9 virus particles, with the T7 RNA polymerase-based reverse-genetics system. Both direct plasmid CVA9, and three different forms of CVA9-eGFP including CVA9-eGFP6, CVA9-eGFP7, and CVA9-eGFP11 plasmid DNA and their T7-tagged PCR amplicons were transfected into BHK-21 and T7-BSR cells using the transfection agent Lipofectamine™ 3000 Reagent. The infection continued for 24-48 hours. 24-48 hours later, the cells were transferred to a new medium for endpoint titration with 10-fold dilution. Another 24 hours later cells were fixed and the cell's nucleus was stained with a DAPI marker. After staining the cells, both T7-BSR and BHK-21 plates were observed for rescuing virus particles. Cells were observed in DAPI mood to assess gross cell morphology

and GFP mood to detect green fluorescence protein released from infected cells. Untransfected cell culture served as a negative control. At 48-h post-infection all the stained wells in T7-BSR plates loaded with the plasmids and PCR amplicons of CVA9 and CVA9-eGFP6, CVA9-eGFP7, and CVA9-eGFP11 samples were observed and green fluorescence assay was visible clearly under the EVOS FL AUTO fluorescence microscope from two wells which was infected by the CVA9-eGFP6 T7-PCR amplicons.

All the plasmids of CVA9 and CVA9-eGFP (s) and other PCR amplicons showed no fluorescence activity. The presence of green fluorescence in wells indicates that on those two particular wells, the CVA9-eGFP6 amplified PCR amplicons successfully infect the host T7-BSR cells. During the cell transfection, the viral DNA was released into host cells for virus replication using the host cell's machinery and the T7 polymerase enzymes produced by the T7-BSR cells. The virus was successfully transcribed into mRNA, and finally, the mRNA was translated to functional proteins such as green fluorescence protein. These two wells were observed under both DAPI and GFP mood and compared with the empty wells (Only medium). The CVA9-eGFP6 genome was amplified with CAV9gen1-R_T7-T^{orig} (Figure 8) regulatory reverse primer infected one well and another well was infected by the samples amplified by the CAV9gen1-R_T7-T^{synt} (Figure 9) regulatory reverse primer in T7-PCR reactions.

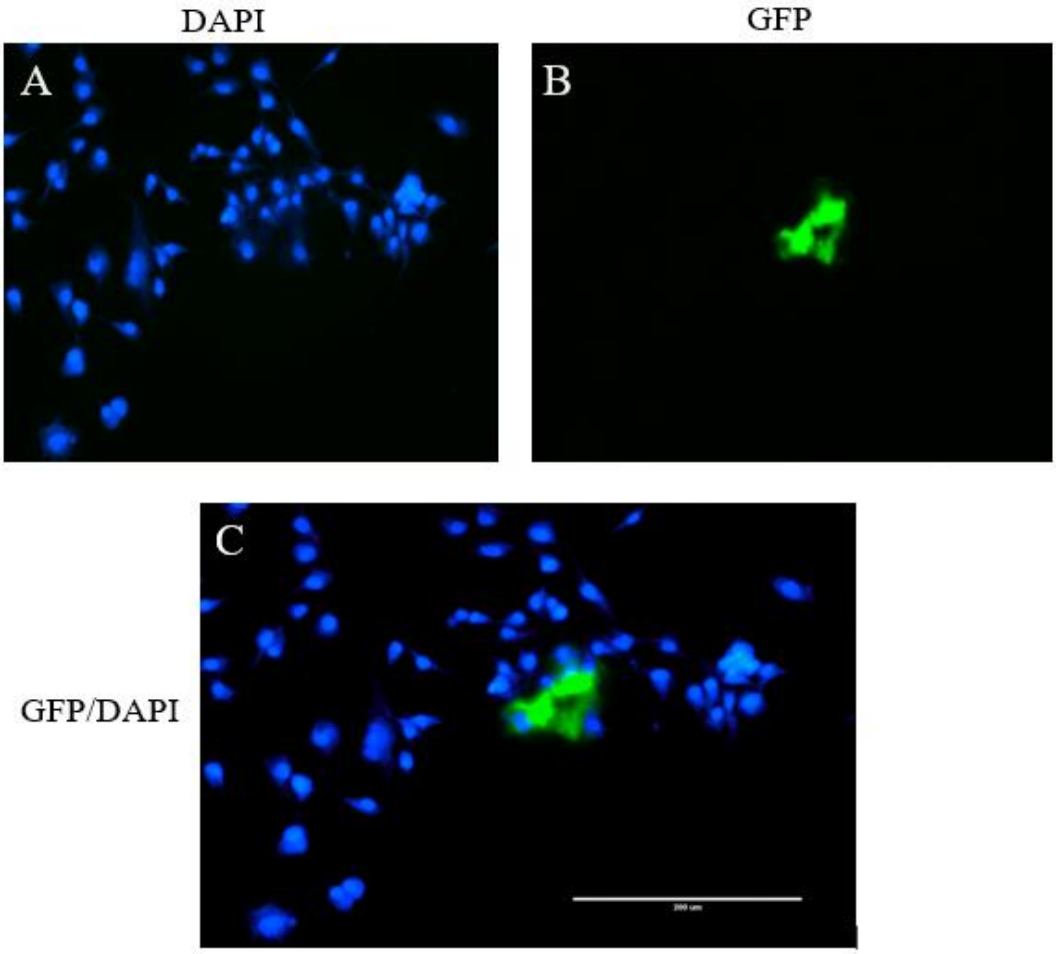

Figure 8 Rescue of the CVA9 infectious virus particles in T7-BSR cells. The T7-BSR cells were transfected with CVA9-eGFP6 T7-tagged PCR amplicons generated by PCR reaction using pCVA9gen1_T7_F forward and CAV9gen1-R_T7-T^{orig} reverse primer. After 24 hours post-infection, the T7-BSR cells were fixed and stained with DAPI (A), the brighter green fluorescence assay indicates the viral mRNA produced green fluorescence protein during replication (B), CVA9-eGFP6 infectious virus particles were detected by green fluorescence assay (C).

On the BHK-21 cells, no green fluorescence assay was detected from any of the wells on the plate. The transfected plasmid DNAs and T7-tagged PCR amplicons did not successfully replicate within the BHK-21 cells. The BHK-21 cells need cotransfection of a T7 RNA polymerase expression plasmid to produce T7 RNA polymerase for viral replication. This BHK-21 cell line served as a cell line control due to their lack of the T7 RNA polymerase enzyme expression system.

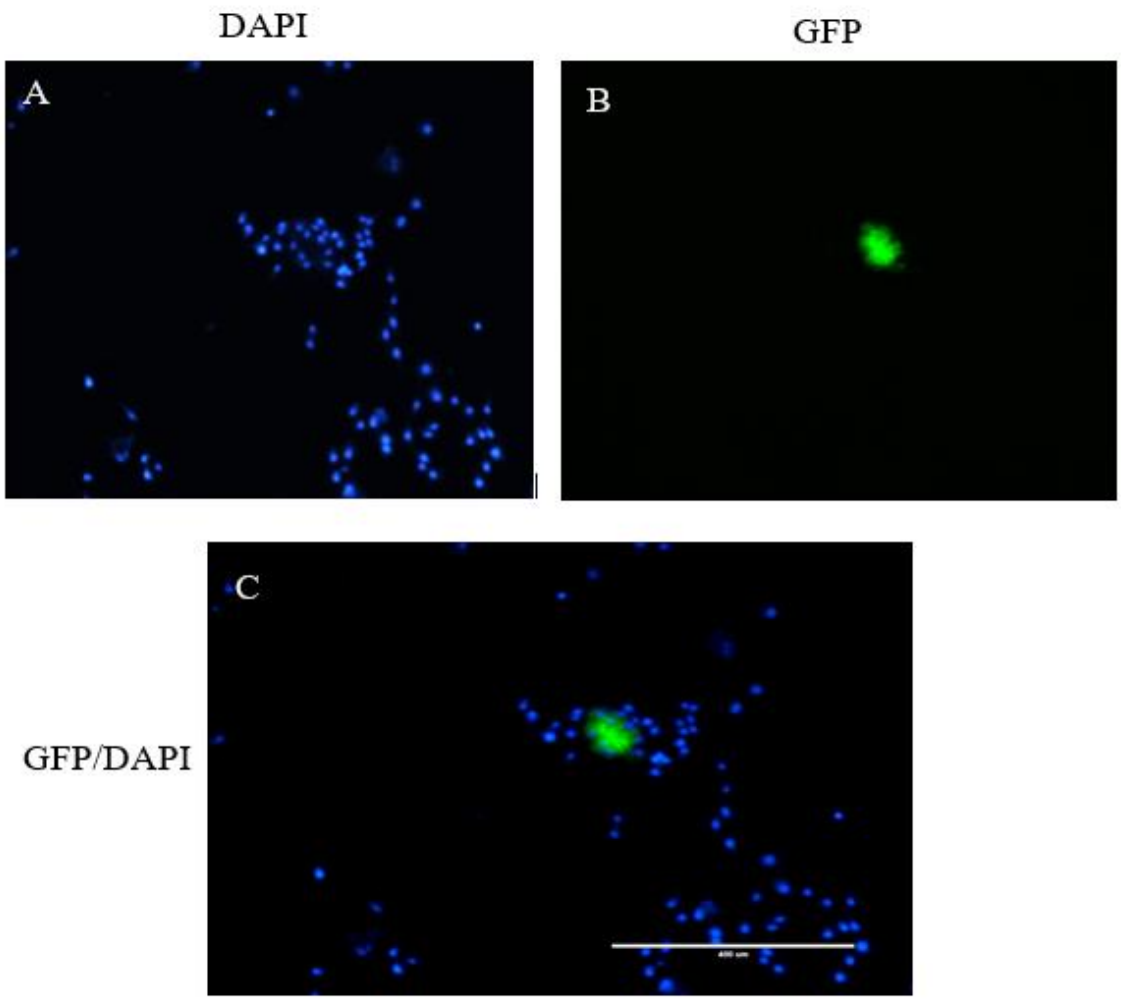

Figure 9 Rescue of coxsackievirus A9 virus particles from T7-BSR cells. Cells were transfected by CVA9-eGFP6 T7-tagged PCR amplicons produced by using CAV9gen1-R_T7-T^{synt} regulatory reverse primer for viral genome amplification. Cell's nucleus fixed and stained with DAPI (A), Green fluorescence assay indicates virus replication (B), GFP/DAPI showed (C) the cytopathic effect due to the formation of virus particles in T7-BSR cells.

We used different plasmid/PCR concentrations versus Lipofectamine™ 3000 Reagent transfection agent (e.g. 1:1, 1:3, and 3:1) to determine the ratio between DNA and transfection agent affects transfection efficiency. However, only the 1:1 ratio of DNA and transfection agent was able to rescue virus particles in those two wells and other ratios were unsuccessful. We conclude that the coxsackievirus A9 (CVA9) using T7 promoter-tagged CVA9 genomic amplicon can successfully utilize this T7 RNA polymerase expressing T7-BSR cells to produce and rescue infectious virus particles using Lipofectamine™ 3000 transfection reagent.

4 Discussion

4.1 Verification and restriction mapping of the plasmid

This study aimed to analyze the functions of genetically modified coxsackievirus A9 (CVA9) bearing green fluorescent protein-encoding gene (CVA9-eGFP). CVA9-eGFP was introduced into adherent cells by transfection and virus growth was monitored by immunofluorescence based on green fluorescence. Initially, several stock preparations of the CVA9 virus with the GFP gene from prior work were verified by restriction analysis. In addition, plasmids encoding T7 RNA polymerase were isolated and verified; these were essential in the conversion of CVA9-eGFP PCR to RNA forms for virus rescue.

The work contained several steps where different obstacles. Initially, there was 7 CVA9-eGFP candidate plasmids were used. These plasmids originated from different prior research projects, and it was essential to identify a single plasmid that contained the GFP insert. Restriction enzyme analysis was important for validating the plasmids because it differentiates isolates based on the restriction site differences and fragments generated by various enzymatic digestions (Wu et al., 2018). There was three different CVA9 without GFP plasmids were used initially, but after restriction enzyme analysis, only one was used for further work as a plasmid control. For restriction analysis of the CVA9 genome FastDigest ClaI enzyme was used with tango buffer (Thermo Fisher Scientific) and using this buffer the downside was it took so much time (1 hour) compared to other FastDigest enzymes for heat inactivation. This issue created problems when working with other CVA9 with GFP samples as they had to wait much more time after their heat inactivation for agarose gel electrophoresis. The extended incubation period with restriction enzyme produced unwanted fragments on the agarose gel, which interpreted results more complicated for verification of the plasmid construct. Two of the CVA9 viral genomes containing plasmids didn't show any good results on gel analysis. To accomplish the aim of the project at the beginning there were also plans for direct co-transfection of the CMV promoter-driven T7 RNA polymerase-producing plasmids (both CMV-T7RNAP and CMV/T7-T7RNAP) with CVA9-eGFP plasmids on A549 cells for generating virus particles. For that reason, the pCMV-T7RNAP and pCMV-T7/T7RNAP plasmids were cultivated and isolated from multiple stocks from previous work. 7 pCMV-T7RNAP plasmids out of 10 plasmids showed promising results after analysis with restriction enzymes, and there were 2 pCMV-T7/T7RNAP plasmids and after analysis, only one pCMV-T7/T7RNAP plasmid showed positive results. The selected plasmid stocks are then cultivated and transformed into E. coli

bacterial strains. The plasmids were isolated and purified from those bacterial cultures using different commercial kits. For isolating larger amounts of DNA several commercial maxi prep kits were used but not all of them worked as the company claimed. The QIAGEN Plasmid Plus Kits were used to isolate high-yield plasmids according to the manufacturer's instructions. They claimed up to 1 mg can be isolated from 200 ml culture volume with a good concentration, while for this experiment 1 mg plasmid DNA was purified with low concentration, and for this reason, plasmids yield more than 500 mg culture volume to obtain more DNA and eventually it was not that much DNA as we expected. So, another maxi prep kit, GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit was used for purifying DNA. From the manufacturer's guide 2.5 ml of purified DNA can be isolated from 150 ml culture volume, so we used 200 ml culture volume to get concentrated purified DNA. These isolated DNA were used as DNA templates for producing T7-CVA9-eGFP-PCR amplicons. For cell transfection appropriate plasmids were crucial for ensuring successful virus rescue making these plasmid preparation steps very accurate and efficient.

4.2 Optimization of T7-promoter-tagged PCR amplicons for virus rescue

The generation of T7-promoter-tagged PCR amplicons was very important for rescuing infectious virus particles through the T7 RNA polymerase-based reverse genetics system. Long-PCR techniques were used in this project to amplify the whole full-length CVA9 viral genome with specific primer sets (Table 3). The forward primer, pCVA9gen1_T7_F designed in such a way as to incorporate the T7-promoter region in the CVA9 genome, and three different regulatory reverse primer sets were tested to how stimulation of T7 by enhancers affects virus transcription. The T7-promoter sequence is designed in a way that the T7 RNA polymerase can recognize and bind to the T7-promoter sequence, and initiates transcription of the downstream CVA9 viral RNA for producing viral RNA transcripts that are important for viral replication and assembling new virus particles (Heikkilä et al., 2011). The T7-promoter in front of the 5´-end of the CVA9 genome was recognized by the T7 RNA polymerase enzyme presented in the mammalian cell line T7-BSR during viral replication. Using different regulatory primers in the PCR reaction allowed to study of the gene expression on viral replication and optimized the PCR process. The CVA9 and CVA9-eGFP (s) viral genome was 7.5 and 8.2 Kb which was a comparatively long genome and amplifying the whole viral was very challenging due to maintaining the throughput. In a longer genome, the throughput is lower compared to the shorter sequence, and to overcome this problem high-fidelity DNA polymerase enzymes were used. Based on previous studies, multiple high-fidelity, and robust PCR enzymes were planned to

generate T7-promoter tagged PCR amplicons to ensure the CVA9 virus replication and transcription by T7 RNA polymerase in the T7-BSR cells. Q5® Hot Start High-Fidelity DNA polymerase (Tan et al., 2016) enzyme was used to amplify EV-A71 cDNA successfully and Phusion Hot Start II DNA polymerase enzyme was successful for amplifying modified IRES fragments of Foot-and-Mouth Disease Virus (Heikkilä et al., 2011; Rai et al., 2015). Eventually, the Platinum SuperFi II DNA polymerase enzyme was used for CVA9 genome amplification, which successfully amplified the CVA9 and CVA9-eGFP (s) viral genome by long-PCR in a previous study (Koskinen, 2023). The agarose gel electrophoresis technique was used for analyzing the PCR products before cleaning up the PCR products. The CVA9 and CVA9-eGFP (s) T7-PCR amplicons generated by forward primer pCVA9gen1_T7_F and reverse primer pCVA9gen1_R were run on agarose gel and showed positive results that indicated the PCR was successful (Figure 7). Other primer sets were also used for producing T7-promoter-tagged PCR amplicons and after gel analysis, those PCR products showed similar results to Figure 7, which are not shown here. This ensured that the T7-PCR amplicons generated through regulatory primer sets were suitable for subsequent transfection experiments to in vivo rescue of infectious CVA9 viral particles.

To amplify a genome using long-PCR reaction depends on several key reaction factors such as primer design, annealing temperatures, or extension periods, any deviation from these factors leads to inconsistent amplification and the production of non-specific PCR products. Amplifying the full-length CVA9 genomes which are 7.5 to 8.4 kb long alongside maintaining the fidelity of long PCR amplicons is challenging and prone to errors. These challenges can lead to the production of incomplete T7-promoter tagged PCR products, ultimately affecting the virus rescue efficiency and replication. Optimizing the long-PCR conditions by exploring different high-fidelity PCR enzymes or more specific primer designs could increase efficiency and specificity. Next-generation sequencing (NGS) of the amplified PCR amplicons could provide us with more accurate and detailed information about the whole CVA9 viral genome, which can be used to develop an improved optimized PCR method for amplifying other larger viral genomes for reverse genetics studies.

4.3 Transfection and *in vivo* virus rescue

The main aim of this current project was to generate CVA9-eGFP virus particles from cDNA clones or T7-promoter-tagged PCR amplicons using a helper virus-free rescue system by transfecting mammalian cells. For this BHK cells containing a stable insert of the T7 RNA polymerase gene (T7-BSR) were used to produce infectious CVA9-eGFP virus particles (Brisson et al., 1999). In previous studies, the T7-promoter-based virus rescue using reverse genetics was done by using in vitro RNA transcripts, but the main disadvantage of this in vitro technique is that these viral particles are less infectious compared to the viral RNA (Boyer and Haenni, 1994; Rieder et al., 1993; Zibert et al., 1990). In some studies, it was proven that the infectious virus particles can be generated from many enteroviral cDNAs and T7-promoter tagged CVA9 PCR amplicons using the T7RNApol-mediated in vivo transcription method (Heikkilä et al., 2011). These T7-BSR cells were already used for generating coxsackievirus A7 (CVA7) virus particles from cDNA successfully (Ylä-Pelto et al., 2016). As CVA9 is a small RNA virus, the T7 RNA polymerase enzyme is crucial for the efficient transcription of viral RNA during viral replication. The CVA9 and CVA9-eGFP full-length viral genomes were amplified under the control of a T7 promoter at the 5´ end for utilizing the T7 RNA polymerase present in T7-BSR cells (Brisson et al., 1999). T7-BSR cells can naturally synthesize T7 RNA polymerase enzyme without any T7RNA encoding helper plasmids, which allows us to bypass the co-transfection of additional plasmids. The BHK cells cannot synthesize the T7 RNA polymerase, so no virus could be generated within these cells, and for this reason, BHK-21 cells were used as a cell line control in this experiment to establish a baseline for transfection efficiency and verifying the viral replication using T7-promoter system. After purification of the T7-promoter-tagged PCR amplicons using PCR purification kits, a cell transfection scheme was prepared for generating virus particles. Direct plasmids and T7-promoter tagged PCR amplicons both of them transfected to the T7-BSR and BHK-21 cells with transfection agent Lipofectamine™ 3000 Reagent. The transfection agent Lipofectamine™ 3000 Reagent was used as it showed the most successful cell transfection in some studies (Cheow et al., 2020; Vanmechelen et al., 2021). Both BHK-21 and T7-BSR cells were transfected several times with different transfection schemes, and not all of them showed any promising results. The CVA9, CVA9-eGFP6, CVA9-eGFP7, and CVA9-eGFP11 both plasmids form and T7-PCR amplicons were transfected and only CVA9-eGFP6 were able to generate virus particles which visualized under the microscope. After getting positive results for CVA9-eGFP6 from previous transfections, a new transfection scheme was planned using only the CVA9-eGFP6 plasmid

and three different forms of T7-CVA9-eGFP6 PCR amplicons, each amplified with three different regulatory primers, to test the effect of these regulatory primers effect on enhancing viral replication. After post-infection end-point-titration was performed to determine the optimal transfection conditions and dilute the infectious virus particles for counting the infection rate.

In the T7-BSR cells green fluorescence activity (GFP) was observed in two wells containing the T7-CVA9-eGFP6 PCR amplicons under the EVOS FL AUTO fluorescence microscope (Figure 8 & 9), indicates T7 RNA polymerase recognized the T7-promoter present in the CVA9-eGFP6 viral genome for successful viral RNA transcription. The CVA9-eGFP6 viral genome amplified by long-PCR technique with forward primer pCVA9gen1_T7_F, and reverse primers pCAV9gen1-R_T7-T^{orig} (Figure 8) and pCAV9gen1-R_T7-T^{synt} (Figure 9) were enhanced the generation of CVA9-eGFP virus particles, while reverse primer pCVA9gen1_R amplified CVA9-eGFP didn't produce any progeny. In BHK-21 cells no green fluorescence protein activity was observed as it was expected before the experiments.

The main obstacle for this experiment was time management. Cultivating and harvesting the cells from the tissue culture plate and then transferring 20,000 cells/well one day before the transfection and after transfection allowed the infection for 24-48 hours, performing end-point-titration and finally fixing the cells for analysis under the microscope took so much time. It makes it very difficult to replicate the same experiment several times. Trying different transfection conditions and using different transfection agents and regulatory primers was aimed while starting the project but not all of them were possible to test due to the short time.

At the beginning of this project, we also aimed to test co-transfection of direct CVA9 and CVA9-eGFP (s) plasmids with the CMV promoter-driven T7 RNA polymerase enzyme (pT7RNP) encoding plasmids including pCMVT7 RNAP and pCMV/T7-T7RNAP in both BHK-21 and A549 cells (Brisson et al., 1999). Both pCMVT7RNAP and pCMV/T7-T7RNAP plasmids were isolated and purified for this co-transfection experiment, but due to time limitations, these two plasmids were never used for co-transfection.

4.4 Optimization of transfection conditions

Optimizing the transfection conditions such as cell confluency, DNA concentrations and transfection reagent for successful virus rescue was crucial. The cells needed to be cultivated

every 3-4 days, as 4 days later they began detaching from the cell culture plates (Freshney, 2010), so cells were collected and cultivated every 4 days later. According to the manufacturer's instructions, 5 µL of DNA sample needs to be mixed with 0.15 µL of Lipofectamine™ 3000 Reagent for each well. In this project, three different DNA/ Lipofectamine™ 3000 Reagent ratios 1:1, 1:3, and 3:1 were tested to find out the most optimized ratio for successful cell transfection. Among these three ratios, only the 1:1 ratio of DNA/ Lipofectamine™ 3000 Reagent generated infectious virus particles successfully, while the other wells transfected with 1:3, 3:1 ratio didn't generate any GFP activity under the microscope. The 1:1 ratio indicated that the conditions where DNA and Lipofectamine™ 3000 Reagent were mixed in the same quantity were ideal for CVA9-eGFP6 to infect the T7-BSR cells properly and replicate within it. While starting the project, it was planned to test different transfection agents to find out the best transfection agent and also to find out the transfection efficacy by counting infection cells/ wells based on GFP activity. However, the original plan was to test several transfection agents to figure out the most successful one and access the GFP-positive cells to quantify transfection efficacy, time constraints restricted the investigation to optimizing the DNA/transfection agent ratio.

4.5 Evolutionary and functional insights

The study of modified virus and their ability to generate viral progeny within a controlled environment provides new insights into understanding viral evolution and adaptation. Manipulating the CVA9 genomes for generating recombinant virus particles using a reverse genetics system gives us enough opportunity to study the evolution at the microscopic level. The restriction mapping and enzymatic analysis for plasmid verification depict how the evolutionary pressures can be studied under a controlled environment (Beaty et al., 2017; Powell, 2019). Studying specific mutations introduced on viral genomes and understanding their effects on viral functionality allow researchers to understand viral biodiversity and fitness that is driven by evolutionary mechanisms.

In this project, reverse genetics is used for studying the functionality of the CVA9 virus which could be used for understanding how genetic changes influence viral fitness, adaptation, and evolution. Manipulating the viral genes and observing the phenotypic changes allow researchers to gain insights into the molecular basis of viral fitness and the genetic configurations that can present selective advantages. This experiment can be used to understand how CVA9 interacts with the host cells, showing the evolutionary dynamics of host specificity

and cross-species transmission. Also, studying the modified viral replication in various cells can give us information about how the adaptation mechanisms they used for emerging new viral strains. Reverse genetics has a huge potential for identifying evolutionary constraints and trade-offs, which can be used for understanding viral evolution and developing strategies for antiviral drugs.

4.6 Future directions and applications

The successful characterization of the coxsackievirus A9-eGFP vector opens up huge possibilities in virological research and developing therapies. The ability to visualize the GFP expression under a microscope enables real-time tracking of virus infection, spread, and behaviour giving us valuable information about viral pathogenesis and host-pathogen interactions. Furthermore, this streamlined process for rescuing viruses bypassing the need for in vitro transcription or without any helper virus and subsequent RNA transfection method made this workflow easy and simple, improving its efficiency and accessibility for research and therapeutic purposes.

The findings of this research give us a strong foundation for exploring the potential of CVA9-eGFP vectors in gene therapy and oncolytic virotherapy shortly (McCarthy et al., 2019). As the enteroviruses can (Shakeel et al., 2013) utilize the host cellular receptors for binding to the host cells, this ability can be used for delivering specific drug molecules, monoclonal antibodies, and other sequences designed for blocking any specific proteins, genes, or signaling pathways that can cause diseases. These oncolytic enteroviruses can bind overexpressed integrin receptors on cancer cells, leading to lysis of the tumor cells without harming the healthy tissues. Another coxsackievirus A11 used as an immunostimulator to stimulate the generation of T cells through robust stimulation of antigen-presenting cells for killing the cancer cells (Sakamoto et al., 2023) showed a promising anticancer strategy. Using the T7-promoter-based PCR amplicons for utilizing the T7 RNA polymerase enzyme-based experiments helps us to establish new routes for developing efficient virus rescue systems which can bring revolutionary advancements in targeted therapy and virus vaccine development. These projects can be used to enhance the development of noble therapeutic agents and vaccine creations with more safety.

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6 List of abbreviations

CVA9	Coxsackievirus A9
cDNA	Complementary DNA
CMV	Human cytomegalovirus
CNS disease	Central nervous system disease
CPE	Cytopathic effect
DPI	Days post-infection
eGFP	Enhanced Green Fluorescent Protein
FDA	Food and Drug Administration
FMDV	Foot-and-mouth disease
IPV	Inactivated polio vaccine
IRES	Internal ribosome entry site
Kb	Kilobase pair
ORF	open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
VPg	Genome linked viral protein

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8 Appendices

8.1 Appendix 1 Supplement links

- Picornavirus taxonomy chart - https://www.picornaviridae.com/
- One Shot TOP10 (E. coli) - https://www.thermofisher.com/order/catalog/product/C404003
- NucleoSpin Plasmid Transfection-grade Miniprep kit manual- https://www.takarabio.com/documents/User%20Manual/NucleoSpin%20Plasmid%20Transfection/NucleoSpin%20Plasmid%20Transfection-grade%20DNA%20Purification%20User%20Manual.pdf
- GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit manual- https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/375/336/na0400sbul.pdf
- NucleoSpin® gel and PCR clean-up kit manual- PCR product purifier- https://www.mn-net.com/media/pdf/02/1a/74/Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf
- GeneRuler 1 kb DNA Ladder- https://www.thermofisher.com/order/catalog/product/SM0311?SID=srch-srp-SM0311
- https://www.denovix.com/products/ds-11-fx-spectrophotometer-fluorometer/
- https://www.fishersci.com/shop/products/viewplate-96-black-50-box/509051605
- https://www.thermofisher.com/fi/en/home/life-science/cell-culture/cell-culture-plastics/cell-culture-flasks/t75-flasks.html

8.2 Appendix 2 Picture

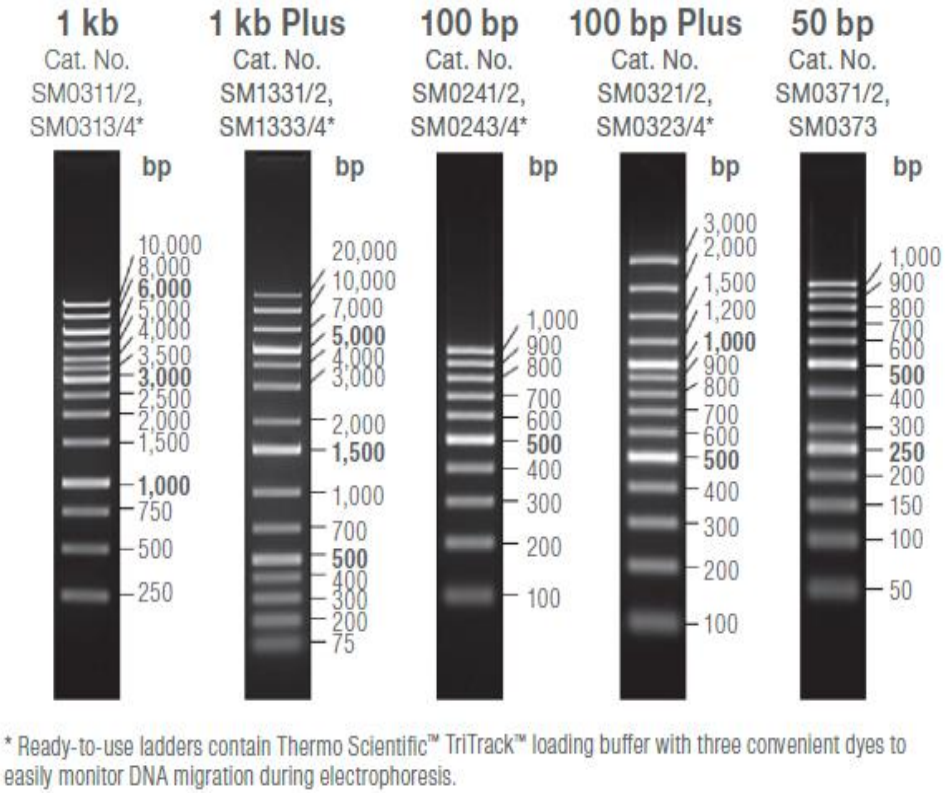

Figure A 1. GeneRuler DNA Ladder Mix.