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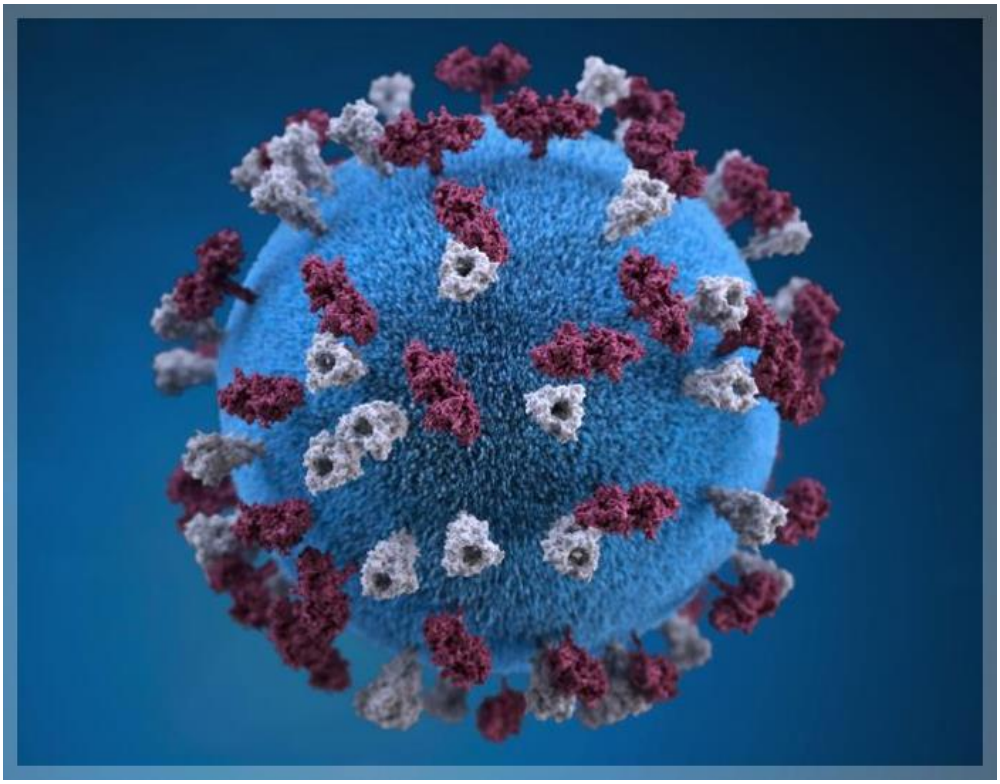
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health

Comparison of the immunomodulatory capabilities of the V and C proteins of different morbilliviruses

Nelman P.S.B. D Ranasinghe A. M.



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Comparison of the immunomodulatory capabilities of the V and C proteins of different morbilliviruses

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Abstract

During a viral infection, when the virus enters into the cytoplasm, different cell types are able to produce interferons (IFN) as a result of interaction between intracellular pattern recognition receptors (PRR) and RNA helicases. This interferon activates and establishes an antiviral state which limits further spread of the virus. Morbilliviruses are highly infectious viruses that spread mainly via direct contact. The genome of morbillivirus contains six genes, translated into eight proteins. The morbillivirus P gene is translated, in addition to the P protein, to V and C proteins. These proteins are directly related to inhibiting type I interferon expression and signalling and thus limit transcription of interferon-stimulated genes (ISGs). Previous studies show that V and C proteins of morbilliviruses have an ability to interfere with the type I IFN pathway at different levels, and the V protein is the main inhibitor. This study focuses on the immunomodulatory capability of a newly discovered *Feline morbillivirus* (FmoPV) in comparison with the well-known *Canine distemper morbillivirus* (CDV) of dogs and *Measles morbillivirus* (MeV) of humans. The V proteins of all the tested viruses strongly inhibited the type I IFN pathway compared to the C proteins. The V and C proteins of FmoPV showed lower immunomodulatory capabilities compared to CDV and MeV. The V protein of CDV showed highest inhibitory ability of tested V proteins and the C protein of the MeV showed the highest inhibitory ability of the tested C proteins. In both cases, significant inhibitory similarities between V and C protein of CDV and MeV were observed.

Keywords: Feline morbillivirus, Canine distemper morbillivirus, Measles morbillivirus, Type I Interferon, C protein, V protein.

Summary

The virus genus known as morbillivirus is highly contagious and spread mostly by direct contact. To date seven species of morbilliviruses have been identified. The most known among them is the *Measles morbillivirus* (MeV), which causes a disease commonly known as measles. Measles is one of the main causes of child mortality around the world. However, a successful vaccination program against the virus reduced the measles deaths by 84% during the years 2000-2016. *Rinderpest morbillivirus* (RPV), which infects cattle, is the second virus eradicated in the world by successful vaccination programs. This virus has, pathogenically, a very close relationship with MeV. The *Canine distemper morbillivirus* (CDV) causes infections in dogs and other carnivores species and it is closely related to *Phocine distemper morbillivirus* (PDV), which causes distemper disease in seals. *Small ruminant morbillivirus* (SRMV) and *Cetacean morbillivirus* (CeMV) are another two morbilliviruses that cause disease in small ruminants and disease in dolphins and whales, respectively. The *Feline morbillivirus* (FmoPV), which causes infections in cats, is the latest identified morbillivirus species among all the seven morbilliviruses.

The morbillivirus genome consists of six genes, namely N, P, M, F, H and L, which encode eight proteins. Interestingly, the P gene encodes two extra proteins, namely V and C protein, in addition to the P protein. These V and C proteins are very important for morbillivirus to fight against the host immune system. These proteins have an ability to inhibit the signalling protein interferon (IFN), which activates the antivirus defence and is produced by the host cells.

This study examined the ability of the V and C proteins of the morbilliviruses FmoPV, CDV and MeV to inhibit IFN and specifically type I IFN. To fulfil this goal, the poly(I:C) stimulated human embryonic kidney (HEK) cells, which mimicked as virus infected cells were transfected with V and C protein plasmids of FmoPV, CDV and MeV. Poly(I:C) is a synthetic double-stranded RNA molecule that mimics a molecular pattern associated with virus infections and activates type I IFN responses. The V and C proteins of FmoPV, CDV and MeV were produced in HEK cells. The ability of these proteins to inhibit type I IFN

levels were measured using reporter system. According to the results, the V proteins of all the tested viruses strongly inhibit type I IFN signalling in contrast to the C proteins. The V and C proteins of FmoPV showed weaker inhibitory capabilities as compared to the corresponding proteins of CDV and MeV. The V protein of CDV showed the highest inhibitory ability of all the tested V proteins and the C protein of the MeV showed the highest ability of all the tested C proteins. This study and its results are helpful for future research regarding morbilliviruses, particularly FmoPV, and vaccine development studies.

Keywords: Feline morbillivirus, Canine distemper morbillivirus, Measles morbillivirus, Type I Interferon, C protein, V protein.

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Abbreviations

CDV	Canine distemper morbillivirus
CemV	Cetacean morbillivirus
CNS	Central nervous system
CTD	Cysteine rich domain
F	Fusion protein
FmoPV	Feline morbillivirus
G	Guanidine
H	Hemagglutinin
HEK 263T	Human Embryonic Kidney 293T
IFN	Interferon
IFNAR1	Interferon alpha receptor subunit 1
IFNAR2	Interferon alpha receptor subunit 2
IRF9	Interferon regulatory factor 9
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK1	Janus kinase 1
L	Polymerase protein
LB	Luria Broth
M	Metrix protein
MDA-5	Melanoma differentiation-associated factor 5
MeV	Measles morbillivirus
MV-Ed	Edmonston B vaccine strain of MeV
N	Nucleocapsid protein
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
P	Phosphoprotein
PDV	Phocine distemper morbillivirus

PLA	Proximity ligation assay
PLB	Passive lysis buffer
Poly(I:C)	Polyinosinic:polycytidylic acid
PPRV	Small ruminant morbillivirus
RLU	Relative luciferase units
RPV	Rinderpest morbillivirus
STAT	Signal transducer and activator of transcription

1 Introduction

Morbilliviruses belong to the family *Paramyxoviridae* and the order of *Mononegavirales*. They are highly infectious and spread mostly by direct contact (Pfeffermann et al., 2018). They are responsible for several severe and moderate distinct diseases, which usually cause long-lasting immunosuppression in humans and animals. There are seven species of morbilliviruses identified until today, namely *Canine distemper morbillivirus* (CDV), *Measles morbillivirus* (MeV), *Small ruminant morbillivirus* (SRMV), *Cetacean morbillivirus* (CeMV), *Phocine distemper morbillivirus* (PDV), *Rinderpest morbillivirus* (RPV) and the recently discovered *Feline morbillivirus* (FmoPV) (de Vries et al., 2015).

1.1 Structure

All the morbilliviruses share the same structural setup. Virions have diameters of about 150 nm and are pleomorphic (Pfeffermann et al., 2018). Morbilliviruses have a non-segmented, negative-sense single-stranded linear RNA genome that contains six genes in the order 3′-N-P/V/C-M-F-H-L-5′ and a set of eight encoded proteins (Figure 1). The lipid envelope of the morbilliviruses contains three viral proteins named Matrix protein (M), Fusion protein (F) and Hemagglutinin (H) (Figure 2). Nucleocapsid protein (N), Phosphoprotein (P) and Polymerase protein (L) (da Fontoura Budaszewski and von Messling, 2016) are the three proteins contained in the ribonuclease protein complex. In addition to P protein, the P gene also encodes two proteins namely V and C proteins (Figure 1). The P protein is a direct mRNA translation of the P gene, while the C protein is generated by initiation of translation at the second AUG of an internal open reading frame (ORF) of the P gene transcripts. Interestingly, the V protein is produced by inserting

a guanidine (G) residue into the mRNA transcript resulting in a switching of the reading frame in the coding sequence (de Vries et al., 2015; Sanz Bernardo et al., 2017). This is called mRNA editing.

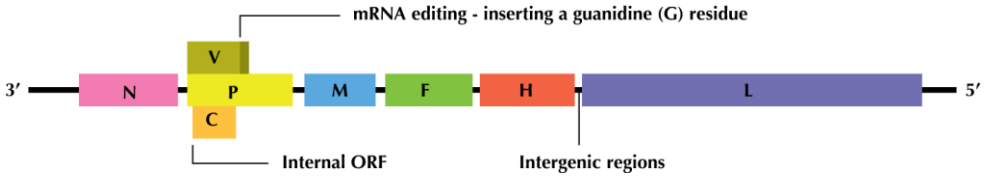

Figure 1. Negative-sense single-stranded linear RNA genome of morbilliviruses. Adapted from Swiss Institute of Bioinformatics (2018).

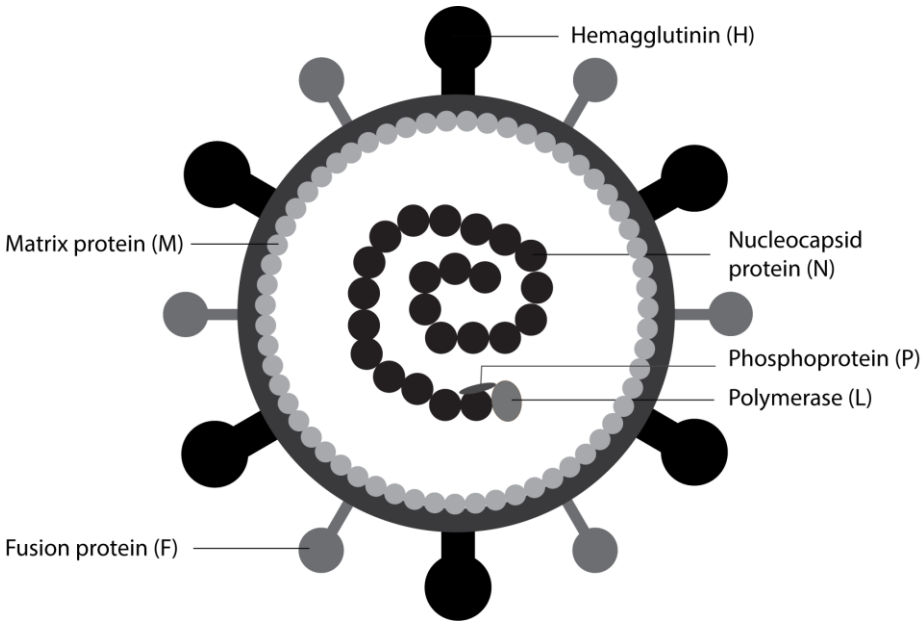

Figure 2. Structure of a morbillivirus. Adapted from Swiss Institute of Bioinformatics (2018).

1.2 Feline morbillivirus (FmoPV)

FmoPV is mainly infecting domestic cats. It was first identified in 2012 in China, Hong Kong and Japan (Woo et al., 2012). Later the same virus was identified from various other places around the world, including Sweden (Dahl, 2018). Phylogenetically FmoPV is related to the other morbilliviruses,

but pathogenically it differs from the others (de Vries et al., 2015). For example, FmoPV has been suggested to be associated with chronic renal failure and tubulointerstitial nephritis which is a disease common in aged cats. However, the other morbilliviruses have not been shown to be associated with tubulointerstitial nephritis. In contrast to the other morbillivirus species, FmoPV is thus mostly associated with problems in the urinary system (Sharp et al., 2016).

1.3 Measles virus (MeV)

Measles has been known for a very long time. MeV is one of the most common morbilliviruses, and infections result in significant levels of morbidity and mortality when humans are infected at an early age. The symptoms of the disease are fever, cough, skin rash and conjunctivitis (de Vries et al., 2015). This disease also causes immune suppression. Severe cases of measles lead to lethal conditions such as gastrointestinal diseases and pneumonia. MeV is different from other morbilliviruses due to the fact that its host range is limited to humans and non-human primates. Therefore, a very successful vaccine has been developed against MeV. However, a minimum of 95% herd immunity is needed to prevent outbreaks of this virus (Fox, 1983). So, even today measles cases are sometimes appearing because some people choose to opt out from being vaccinated due to economic, cultural and political beliefs.

1.4 Canine distemper virus (CDV)

The canine distemper disease has been known since the 17th century. It is a disease affecting dogs and a broad range of other carnivorous animals causing a very high rate of morbidity and mortality. Due to the severity of the disease, most domestic carnivores are vaccinated against this virus (de Vries et al., 2015). The CDV infection impacts the gastrointestinal, respiratory and central nervous systems very seriously. The symptoms of the disease vary based on the age, immune state of the animal and strain of the virus (Beineke et al., 2015).

1.5 Virus infections and interferon (IFN) responses

When an infection from a virus occurs, the host innate immune responses are activated by the recognition of viral pathogen-associated molecular patterns (PAMPs) via cellular pattern recognition receptors (PRRs) (González-Navajas et al., 2012). This recognition activates the intracellular signalling pathway that leads to the production of interferons (IFNs) one of a number of pro-inflammatory cytokines. A virus-resistant state in cells is later induced by IFN α/β. Also, these are highly important for later adaptive immunity responses.

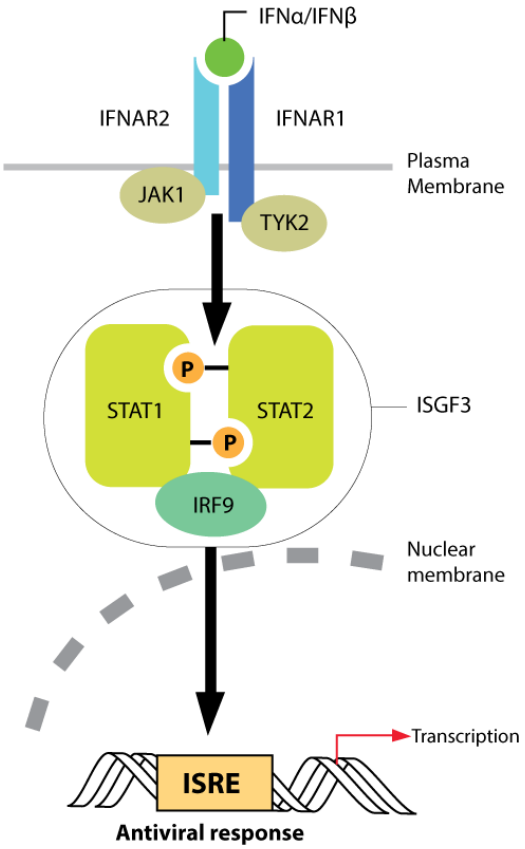

Figure 3. Virus infections and type I IFN responses. Adapted from González-Navajas et al. (2012).

As shown in Figure 3, the IFN α/β bind to the interferon-α receptor that is composed of two subunits, interferon alpha receptor subunit 1 and 2 (IFNAR1/IFNAR2) (Pfeffermann et al., 2018). This binding activates two

members of this pathway, Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) (Pfeffermann et al., 2018). As a result of the phosphorylation by these two kinases, signal transducer and activator of transcription (STAT) proteins are recruited to the pathway. There are three different STAT proteins (STAT1, STAT2 and STAT3) that are of importance in viral infections. STAT1 and STAT2 proteins are subjected to phosphorylation and form a heterodimer complex, together with interferon regulatory factor 9 (IRF-9), a DNA binding protein (González-Navajas et al., 2012). This heterodimer complex is known as interferon stimulated gene factor 3 (ISGF3). Thereafter, ISGF3 translocates to the nucleus from the cytoplasm and binds to interferon stimulated genes (ISGs). The binding site is located in the promoter region of the ISG gene, a specific conserved nucleotide sequence of about 15 bp size known as interferon stimulated response element (ISRE) (Chinnakannan et al., 2013). Activation of ISG gene transcription induces the expression of a large number of proteins and ultimately leads to an antiviral state of the cell.

1.6 Strategies of type I IFN inhibition of paramyxoviruses

In general viruses of the family of *Paramyxoviridae* use different strategies to inhibit the type I IFN pathway. The results obtained from previous studies show that the majority of the viruses from the genera *Rubulavirus* and *Avulavirus* use the strategy of degradation of STAT proteins for their immunomodulation (Chinnakannan et al., 2013). On the other hand, viruses of the genera *Morbillivirus, Henipavirus* and *Respirovirus* are not involved in degradation of STAT proteins, and instead these viruses inhibit the phosphorylation of STAT proteins or nuclear accumulation. Even the immunomodulatory capability of the V and C proteins differ between genera. The V protein of viruses of the genera *Henipavirus* and *Morbillivirus* blocks IFN signalling, while the C protein of respiroviruses blocks IFN signalling. (Chinnakannan et al., 2013).

1.7 Morbillivirus V and C proteins

The V and C protein of morbilliviruses, known as non-structural or alternative proteins, are encoded by the above-mentioned P gene. The V protein is larger than the C protein for every species of morbillivirus. Even

the V proteins have more versatile immunomodulatory characteristics than the C protein. The V and C proteins of morbilliviruses also play a major role in transcription and translation during the viral life cycle (Su et al., 2015). The evasion of the interferon response by the V protein of morbilliviruses is mediated by the V-specific domains consisting of a highly conserved motif that contains seven cysteine residues (Fontana et al., 2008). Different morbilliviruses use different blocking mechanisms to inhibit the type I and type II interferon pathways (Chinnakannan et al., 2013). The V protein of all morbilliviruses can interfere with STAT and STAT2 components of the host's IFN response pathway (Svitek et al., 2014) and the result of this will lead to the inhibition of the STAT nuclear translocation (Horvath, 2004). Furthermore, previous studies have shown that if the V and C proteins are defective MeV replication is reduced and those MeV viruses have a shorter life span compared to the wild type (Devaux et al., 2008). In the IFN pathways, the V proteins of morbilliviruses form a complex with TYK2 and JAK1. The newly formed complex has the ability to inhibit phosphorylation of STAT1 and STAT2 (Chinnakannan et al., 2013).

The C protein studies are mostly related to different wild type and vaccine strains of MeV. Previous studies have shown that the C protein has the ability to downregulate viral polymerase activity. Also, it increases the production of the C protein of MeV and reduces the IFNα/β production of infected cells. Further, it also enhances the viral activity that inhibits IFNα/β signalling (Shaffer et al., 2003). For viruses that lack or have defective C protein, viral RNA synthesis results in highly stimulated IFN production (Nakatsu et al., 2008). Further studies have also shown that C protein of MeV binds to STAT1 and inhibits the phosphorylated dimerization activity of the pathway (Yokota et al., 2011).

1.8 V and C proteins of FmoPV

The V and C proteins of the newly discovered FmoPV are apparently different from those of other morbilliviruses. FmoPV has the largest genome size, 16050 nt, of all the morbilliviruses (CDV consists of 15690 nt and MeV consists of 15894 nt). Even the amino acid length of the V and C proteins of FmoPV, 276 and 170 respectively, is different from the V and C proteins of CDV, 299 and 174, respectively, and MeV, 299 and 186, respectively (Figures 4 and 5).

Therefore, it is interesting to investigate the immunomodulatory capabilities of the V and C proteins of FmoPV in comparison to these other viruses. Such a comparison has not previously been conducted, specifically a systematic comparison between FmoPV, CDV and MeV.

1.9 The main goal of study

The main goal of this thesis was to investigate the ability of V and C proteins of FmoPV to inhibit induction of immune response by type I interferon and compare it to other related morbilliviruses such as MeV and CDV.

2 Methods

The thesis aims at understanding how the type I IFN inhibition of V and C proteins of FmoPV differ from that of V and C proteins of CDV and MeV. In doing so, several methods in bioinformatics, cloning of viral genes, transfection of proteins and measuring type I IFN levels using reporter system were being used. Several protocols available at the manufacture's Thermo Fisher, Mirus, Promega and Tecan were used for these experiments.

Bioinformatics methods were used, as described below, for obtaining the cDNA sequences of V and C proteins from whole genomes. This was followed by multiple sequence alignment analysis of proteins of FmoPV, CDV and MeV (Figures 4 and 5). Cloning methods were used for the expression of viral genes. The relative immunomodulatory capabilities of the V and C proteins of FmoPV, CDV and MeV in inhibiting type I IFN signalling were measured by a modified dual luciferase assay reporter system using the expression of an ISRE-responsive reporter plasmid. The detailed experimental setup is shown below.

2.1 Construction of V and C expression plasmid from FmoPV, CDV and MeV

The V and C plasmid vectors (pCMV-3Tag-1a) of FmoPV, CDV and MeV were constructed at GenScript Inc (Appendix 1). The sequences used for *de novo* gene synthesis of C and V from full genome sequences were available at National Center for Biotechnology Information (NCBI) GenBank: FmoPV strain US1, complete genome – Accession number: KR014147;

CDV isolate CDV2784/2013, complete genome Accession number: KF914669.1; MeV isolate 97-45881, complete genome – Accession number: DQ227319.1. ***FmoPV (V and C; cDNAs):*** For the expression of the C protein, the P gene was used (nt 1812 - 2324 of the genome sequence), while for the V protein the same gene was used, but with the addition of an extra G at nt position 2460 and a different length of the gene (nt 1781 – 2610). As restriction sites, EcoRI at the 5' end and HindIII at the 3' end were added for both cDNAs (Appendix 1 - Figure 1). ***CDV (V and C; cDNAs):*** For the expression of the C protein, the P gene was used (nt 1823 -2347 of the genome sequence), while for the V protein the same gene was used, but with the addition of an extra G at nt position 2493 and a different length of the gene (nt 1801– 2699). The restriction sites EcoRI at the 5'end and HindIII at the 3' end were added for both cDNAs (Appendix 1 - Figure 2). ***MeV (V and C; cDNAs):*** For the expression of the C protein, the P gene was used (nt 1829 -2389 of the genome sequence), while for the V protein the same gene was used, but with the addition of an extra G at nt position 2498 and a different length of the gene (nt 1781 – 2610). As restriction sites, BamHI at the 5'end and EcoRI at the 3'end were added for both cDNAs (Appendix 1 - Figure 3).

2.2 Analysis of V and C genes and proteins of FmoPV, CDV and MeV

The V and C proteins of FmoPV, CDV and MeV were analysed using the Clustal O multiple sequence alignment tool. The sequences of each V and C protein were obtained from the NCBI GenBank sequences of FmoPV, CDV and MeV described in section 2.1.

2.3 Transformation of vector plasmids

The vector plasmids were transformed into Sub-cloning Efficiency™ DH5α™ Competent Cells (Invitrogen Inc.) according to the manufacturer's protocol. A 30 μl aliquot of *Escherichia coli* DH5α cells and 1 μl (1 ng) of the V or C plasmids were used. The plasmid solution and *E. coli* DH5α cells were thawed on ice. Then 1 μl (1 ng) of plasmid solution was added to the

cells followed by 30 minutes of incubation on ice. The tubes were heat shocked at 42°C for 20 seconds and placed on ice for 2 minutes. The cells with plasmids were added to 500 μl of pre-warmed Luria Broth (LB) media tubes and incubated at 37°C for 1 hour with shaking (225 rpm). After incubation, 20 μl to 100 μl of the transformed cells was spread on pre-warmed agar plates containing 50 μg/ml kanamycin. The plates were incubated overnight at 37°C. Next day, a single colony was picked from the agar plate and transferred to 1.5 ml of LB medium with kanamycin and the bacterial culture was incubated at 37°C for 8 hours at with shaking (225 rpm). An aliquot of 100 μl was inoculated to a flask containing 200 ml of LB growth medium with 50 μg/ml kanamycin. The flasks were incubated at 37°C for 16 – 21 hours at 225 rpm. The ISRE luciferase reporter vector plasmid, renilla luciferase vector plasmid and empty vector pcDNA were also transformed following the same protocol, but using 100 μg/ml ampicillin as the antibiotic.

2.4 Plasmid isolation

PureYield™ Plasmid Maxiprep System (Promega) was used for isolating plasmids according to the manufacturer's protocol. The transformed *E. coli* cells were pelleted by centrifugation for 30 minutes at 5000 x g in room temperature. The supernatant was discarded and the pellet was resuspended in 12 ml of cell resuspension solution. Cell lysis solution (12 ml) was added to the tube and the solution was gently mixed 3-4 times followed by an incubation period of three minutes at room temperature. The neutralization solution (12 ml) was added and the tube was gently inverted 10 – 15 times to mix the solution. The lysate was centrifuged at 7000 x g for 30 minutes at room temperature. The lysate was filtered and added to the PureYield™ Plasmid Maxiprep column according to the manufacturer's protocol. The concentrations of the purified plasmids were measured using Qubit 2.0 (Thermo Fisher Scientific).

2.5 Cell line - Human Embryonic Kidney 293T (HEK293T)

The HEK293T cells were grown in serum-containing cell culture medium with 1% penicillin and 10% fetal bovine serum. The HEK293T cells were kept in an incubator at 37°C and with 5% CO_2 for optimal conditions of growth.

2.6 Dual luciferase assay

The HEK293T cells were transfected according to the Mirus Bio TransIT-LTI protocol using 24-well plates. The cells were plated 18-24 hours prior to the transfection with a 70-80% confluency. The seeding density of the cells was 0.05×10^6 and a cell confluency of 0.2×10^6 was maintained prior to the transfection. The previously prepared V and C protein plasmids at concentrations of 250 ng/ml, 500 ng/ml and 1000 ng/ml (final concentration) were used for all the experiments. For the transfection, ISRE-Luc reporter plasmid at a concentration of 200 ng/ml, renilla at 2 ng/ml and empty vector pcDNA at 1000 ng/ml were used. The same concentrations of ISRE-Luc, renilla and pcDNA were used for each control. TransIT-LTI reagent and Opti-MEM reduced-serum medium were used as described in the Mirus Bio protocol (experimental setup shown in Appendix 2). A summary of the protocol is shown below.

The Opti-MEM reduced-serum medium, plasmids, pcDNA, ISRE-Luc and renilla were added to the micro-centrifuge tube. After adding TransIT-LTI reagent to the tube, it was incubated for 20 minutes at room temperature. The calculated amount of reagent and plasmid mixture was added to the wells of the 24-well plate in a drop-wise manner. The plates were incubated at 37˚C for 18 – 24 hours. The next day, the Lipofectamine 3000 reagent was used for enhancing the transfection performance and reproducible results. The viral infection in HEK 263T cells was mimicked using poly(I:C) (1 μg /ml). The poly(I:C) was added for only half of the wells of each concentration and the other half of the wells was used as controls. Poly(I:C) is a double-stranded RNA molecule that is made synthetically, and mimics the molecular pattern associated with virus infections activating type I IFN responses via the melanoma differentiation-associated factor 5 (MDA-5) pathway (Démoulins et al., 2009). After 24 hours of incubation at 37˚C, the growth medium was removed from the wells and cells were lysed using 100 µl of 1X passive lysis buffer (PLB). Then 10 µl of cell lysis solution from each well was added to a 96-well microplate for luminescence reading. Luciferase activity was measured by using an Infinity M1000 microplate reader (Tecan) according to the manufacturer's protocol (Promega). The experiments were done twice with triplicates and all the values were normalised according to the control without poly(I:C). Relative luciferase expression was calculated as a percentage compared to the empty vector, pcDNA control.

3 Results

3.1 Analysis of V and C genes and proteins of FmoPV, CDV and MeV

The purpose of the multiple sequence alignment of V and C proteins is to identify the conserved amino acids region of FmoPV, CDV and MeV. All the V-specific domains of FmoPV, CDV and MeV contains a highly conserved motif of seven cysteine residues (Figure 4). These unique conserved motifs are responsible for binding to zinc molecules. The amino acid lengths of the V proteins of CDV and MeV are the same, but the V protein of FmoPV is shorter than the others.

Figure 4. Multiple sequence alignment of V proteins of FmoPV, CDV and MeV. The seven highly conserved cysteine residues are highlighted in purple. The other conserved amino acids are also highlighted. The amino acid lengths of the V proteins of FmoPV; 276, CDV; 299 and MeV; 299.

The C proteins of FmoPV, CDV and MeV do not contain a highly conserved motif like V proteins. The C proteins of FmoPV, CDV and MeV are shorter in length and differ in amino acid sequence compared to the V proteins.

Figure 5. Multiple sequence alignment of C proteins of FmoPV, CDV and MeV. The amino acid lengths of the C proteins of FmoPV, CDV and MeV are 170, 174 and 186, respectively.

3.2 Inhibition of luciferase expression by V and C proteins of FmoPV, CDV and MeV

The relative immunomodulatory capabilities (inhibiting type I IFN signaling) of the V and C proteins of FmoPV, CDV and MeV were measured according to the expression of ISRE responsive reporter gene on the plasmid using dual-luciferase assay. The normalised data shows the averages of two experiments.

3.3 V proteins of CDV and MeV showed higher immunomodulatory capability than FmoPV

Studies on the level of inhibition of ISRE reporter plasmid expression with different concentrations of V protein plasmids produced the following results. The level of inhibition by the V protein plasmid of FmoPV, CDV and MeV are calculated as a percentage, compared to the empty plasmid vector, pcDNA control (Figure 6). Expression of the V protein of FmoPV at a low

level (250 ng/ml plasmid for transfection) resulted in a reduced expression (59.71%) of the reporter gene. Higher expression of the V protein gave a stronger inhibition of the reporter gene, with 42.59% relative luciferase expression when 500 ng/ml plasmid concentration was used for transfection and 39.83% relative luciferase expression with 1000 ng/ml of plasmid concentration.

The V proteins of CDV and MeV had significantly higher inhibition than the V protein of FmoPV (Figure 6). The relative luciferase expression in the presence of CDV V protein was 13.38% (250 ng/ml), 8.95 % (500 ng/ml) and 9.38% (1000 ng/ml). A similar high level of inhibition was observed for V protein of MeV with relative luciferase expression of 17.06%, 14.94% and 14.45% with respect to the given concentration of V protein plasmids. Interestingly, the V proteins of CDV and MeV were observed to have very similar concentration dependent inhibitory ability.

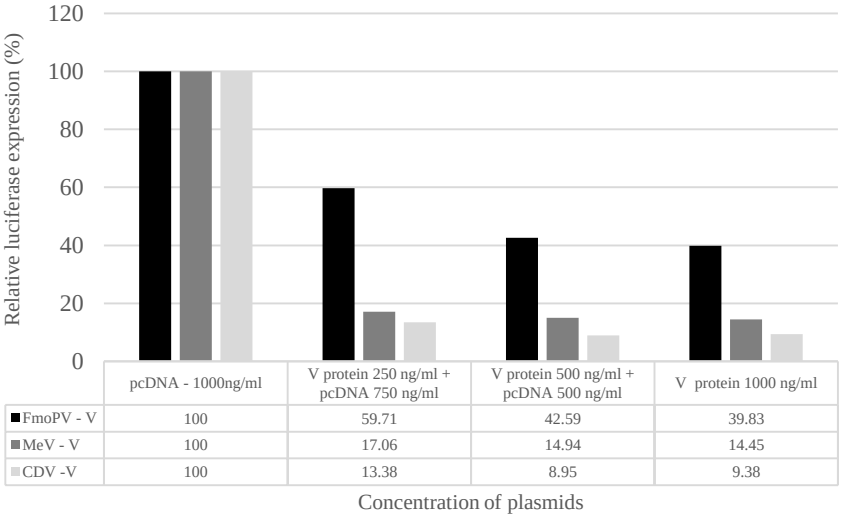

Figure 6. A comparison of the ability of V proteins (FmoPV, MeV and CDV) to inhibit the type I IFN response induced by poly(I:C) stimulation.

Figure 6 also presents a direct comparison of the inhibitory effect of the V proteins of FmoPV, CDV and MeV on type I IFN-induced expression. The V protein of CDV has a higher immunomodulatory capability than the other V proteins, leading to a reduction to 10% of the expression in the control. The V protein of FmoPV has the lowest inhibition of IFN pathway followed by V protein of MeV.

3.4 The C proteins of FmoPV, CDV and MeV show very similar and reduced capability to inhibit the induced response to type I IFN

The C proteins of FmoPV, MeV and CDV showed relatively similar but reduced type I IFN inhibition compared to the V proteins (Figure 7). The relative luciferase expression induced by poly(I:C) was reduced in the presence of FmoPV C protein: 73.11% (250 ng/ml of plasmid used for transfection), 53.17% (500 ng/ml) and 42.33% (1000 ng/ml) compared to the control. These results indicated very clear concentration dependent inhibition by the C protein.

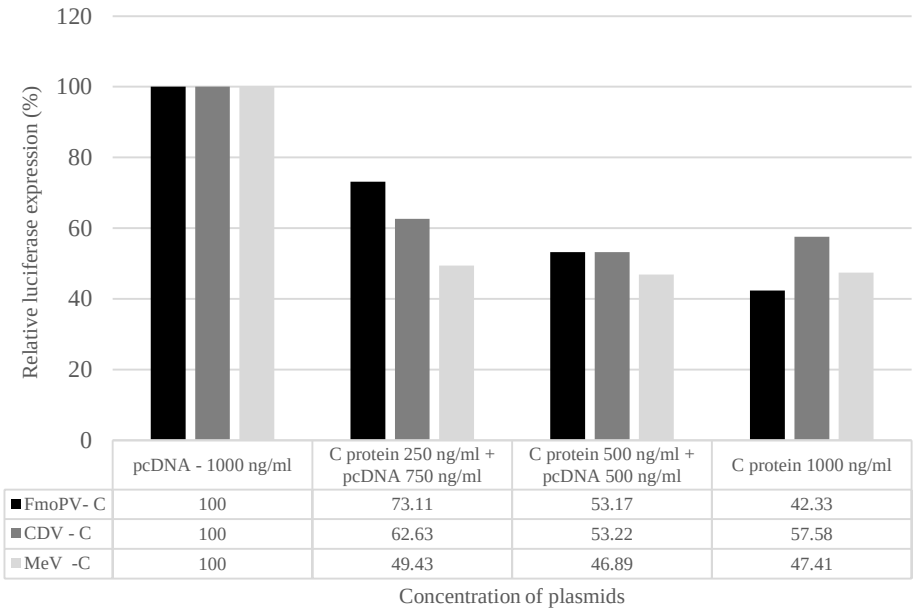

Figure 7. A comparison of the ability of C proteins (FmoPV, CDV and MeV) to inhibit the type 1 IFN response induced by poly(I:C) stimulation.

Interestingly, the results showed that the C proteins of CDV and MeV have very similar capability of inhibition as the C protein of FmoPV. For C proteins of CDV and MeV, the increasing plasmid concentration did not affect inhibition of type I IFN induced expression (Figure 7). The relative luciferase values for the C protein of CDV were 62.63%, 53.22% and 57.58% with respect to the different plasmid concentrations used for transfection. A similar result was obtained for the C protein of MeV with 49.43%, 46.89 and 47.41% of relative luciferase expression for the different concentrations of

plasmids. These results suggest that the C proteins of CDV and MeV have a similar ability to inhibit the type I IFN pathway at different protein concentrations. The C protein of FmoPV shows a clear correlation between increasing concentrations of plasmid for C protein expression and inhibition of type I IFN-induced expression.

3.5 Comparison of type I IFN signalling inhibition by the V and C proteins

The cumulative comparison of all the results for type I IFN signalling inhibition by the V and C proteins of FmoPV, CDV and MeV is shown in Figure 8. It is clear from this graph that V and C proteins of all the tested morbilliviruses have potent inhibition capability.

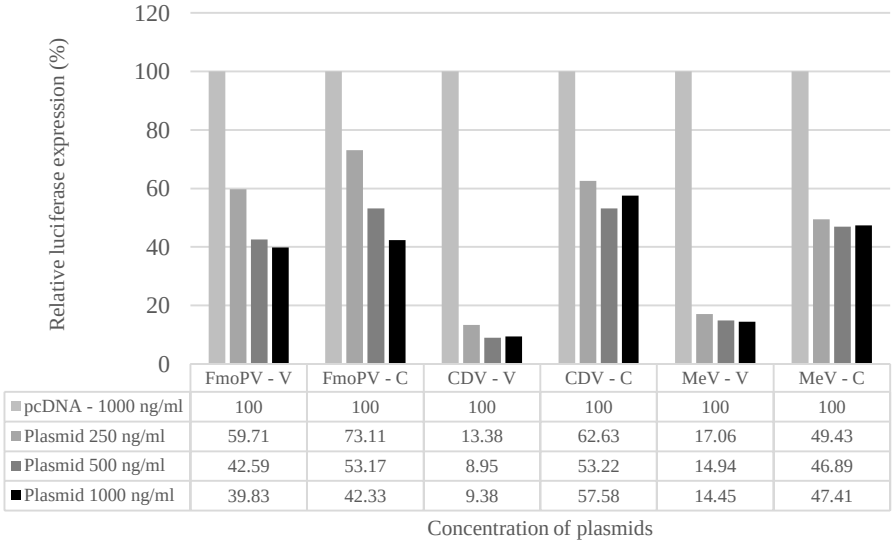

Figure 8. A comparison of the ability of V and C proteins of FmoPV, CDV and MeV to inhibit type I IFN signalling induced by poly(I:C) stimulation.

4 Discussion

Morbilliviruses are highly infectious, mostly spread through direct contact and are responsible for severe and moderate distinct diseases (de Vries et al., 2015). According to previous studies all morbilliviruses initially target the immune system of the host, especially the type I IFN pathway, before spreading to the other cells of the body (de Vries et al., 2015). Therefore, a better understanding of the immunomodulatory capabilities of these viruses is important for the control of these highly infectious viruses. As outlined in the introduction, the V and C proteins of morbilliviruses are responsible for the down-regulation of type I IFN signalling at different levels. Studies have proven that the V and C proteins of these viruses are responsible for blocking different steps of this pathway (Pfeffermann et al., 2018).

Previous studies related to the V and C proteins of the recently identified FmoPV have shown that it is structurally and pathogenically different from other morbilliviruses (Woo et al., 2012). This is the first study focusing on comparing the immunomodulatory capabilities of V and C proteins of the three morbilliviruses FmoPV, CDV and MeV.

4.1 The V and C proteins of FmoPV, CDV and MeV reduce type I IFN induced gene transcription to different levels

This study observed that the V and C proteins of FmoPV, CDV and MeV reduced the type I IFN induced gene transcription to different levels. Studies on the immunomodulatory effects of V and C proteins (Fontana et al., 2008; Sparrer et al., 2012; Chinnakannan et al., 2013) are available for MeV and CDV, but there is a lack of results for the V and C proteins of

FmoPV. The previous findings related to the ability of the V and C proteins of MeV and CDV to inhibit IFN-induced signalling are varying according to the virus isolate and cell type used in the experiments (Fontana et al., 2008). However, all the available results have shown that the V and C proteins of MeV and CDV inhibit type I IFN signalling. According to the study on the V and C proteins of MeV (Fontana et al., 2008), the V protein has a stronger inhibitory effect on signalling than the C protein. Furthermore, the studies of Sparrer et al. (2012) have shown that the C proteins of wild type and vaccine strains of MeV have different capability to inhibit type I IFN signalling. The V and C proteins of MeV used in this study were obtained from the isolate 97-4588, which is a wild-type isolate found in the USA. However, Shaffer et al. (2003) and Ohno et al. (2004) have reported different observations for the inhibition ability of V and C proteins of wild-type and attenuated strains of MeV, due to differences in viral strains and cell types used.

4.2 The V protein of FmoPV, CDV and MeV has stronger immunomodulatory capabilities than the C protein

As outlined in the introduction, the V protein of morbilliviruses has higher immunomodulatory capabilities than C proteins. This study also shows the same observations for all the tested morbilliviruses. The V protein of CDV and MeV inhibited type I IFN signalling with greater efficiency than the C protein of the same morbilliviruses. In a previous study, a similar inhibitory ability of type I IFN signalling by the V protein of these morbilliviruses was reported (Chinnakannan et al., 2013). These findings were also similar to a previous study using recombinant knockout of the highly virulent CDV strain A75/17, where the V protein was shown to be the main antagonist player when it comes to inhibiting IFNα/β signalling and the C protein potently inhibited the activation of the ISRE promoter (Röthlisberger et al., 2010).

4.3 The specific immunomodulatory ability of the V protein of FmoPV

The V and C proteins of FmoPV demonstrated different immunomodulatory abilities compared to those of the other tested morbilliviruses. The V proteins of CDV and MeV strongly inhibited type I IFN signalling, while the V protein of FmoPV did not show similar inhibition. Interestingly, the C protein of FmoPV showed clear concentration-dependent inhibition of type I IFN signalling, while the C protein of the two other tested morbilliviruses gave similar reductions in response for all tested concentrations. Available literature has also shown similar dose-independent inhibitory effects for C proteins of CDV and MeV (Chinnakannan et al., 2013; Fontana et al., 2008).

4.4 Pathological differences of V and C proteins of FmoPV, CDV and MeV

The morbilliviruses used for analysis of V and C proteins belong to wild-type strains that cause severe infections. A previous study has shown that V and C proteins of wild type strains of morbilliviruses have higher capability to inhibit type I IFN signalling than attenuated strains (Sanz Bernardo et al., 2017). Patterson et al. (2000) have shown, by using transgenic mice infected with MeV, that the V and C proteins of MeV act as virulence factors and as a result, the mortality rate of mice infected with V gene defective MeV (V-) was significantly reduced compared to the mortality of mice infected with the Edmonston B vaccine strain of MeV (MV-Ed). The same study also showed that the spread of disease in mice infected by C gene defective MeV (C- MeV) was similar to MeV-Ed but with a lower motility rate. Another study illustrated how V proteins of wild-type MeV and CDV blocked type I IFN inhibition more effectively than the V proteins of the vaccine strain of MeV-Ed and the laboratory strain CDV-Ond (Chinnakannan et al., 2013). When the V and C proteins bind, the antiviral effect of the type I IFN response is reduced (Chinnakannan et al., 2013). Therefore, there is a significant relationship between pathology and inhibition of type I IFN response by V and C proteins. We could assume that V proteins of CDV and MeV strongly inhibit type I IFN signalling, explaining that their spread is faster and virulence is higher than for FmoPV. Compared to the V protein,

the C protein of morbilliviruses showed reduced ability to inhibit type I IFN signalling indicating that those are not as pathogenically strong as the V proteins.

4.5 Protein analysis and immunomodulatory capabilities of FmoPV, CDV and MeV

The V-specific domain of FmoPV, CDV and MeV consists of highly conserved amino acids forming a motif that contains seven cysteine residues. As outlined in the introduction, these highly conserved amino acids are important for the abilities of V proteins of CDV and MeV to reduce the interferon response. The results obtained from this study also showed that the V protein of these morbilliviruses has stronger inhibitory abilities than the C protein. Further studies are required to compare these abilities with those of V and C protein of FmoPV.

4.6 Limitations and future studies

The study presented in this thesis is the first to compare the immunomodulatory capabilities of the concerned morbilliviruses. According to time and other constraints, the inhibition of ISRE promoter gene expression of the type I IFN pathway was the only focus of the experiments. Therefore, this study could be extended to test previously identified other inhibitory points of the pathway RIG-I/MDA-5, JAK1/TYK2, STAT1/STAT2 and IFNAR1/IFNAR2 and compare the inhibition by V and C proteins specifically. Also, the effects of mutations of V and C proteins of FmoPV on their ability to inhibit signalling can be further tested. This could be a possibility to check the immunomodulatory capabilities of vaccine and wild-type strains of morbilliviruses, especially of FmoPV.

This experiment could also be complemented by investigating the protein concentrations and distributions using western blot (protein immunoblot) and *in situ* proximity ligation assay (PLA), respectively. Such investigations would be used to determine the expression of the V and C proteins and to verify these results. These analyses were opted out from the current study due to time restrictions.

5 Concluding remarks

The immunomodulatory capabilities of the V and C proteins of FmoPV, CDV and MeV were examined in this study. The ability of V and C proteins of FmoPV, CDV and MeV in inhibiting type I IFN signalling was measured by the expression of an ISRE responsive reporter using a dual luciferase assay reporter system. The relative luciferase expression was used to analyse the immunomodulatory capabilities of each virus protein.

The conclusion of this study is that the V and C proteins of FmoPV demonstrate a relatively low level of inhibition of type I IFN signalling compared to the V and C proteins of CDV and MeV. The V protein of CDV showed the highest inhibition and the C protein of MeV. Further studies are, however, required to understand better the ability of V and C proteins to inhibit the type I IFN pathway, especially that of the V and C proteins of FmoPV.

References

Beineke, A., Baumgärtner, W., Wohlsein, P., 2015. Cross-species transmission of canine distemper virus-an update. One Health 1, 49–59. https://doi.org/10.1016/j.onehlt.2015.09.002

Chinnakannan, S.K., Nanda, S.K., Baron, M.D., 2013. Morbillivirus V proteins exhibit multiple mechanisms to block type 1 and type 2 interferon signalling pathways. PLOS ONE 8, e57063. https://doi.org/10.1371/journal.pone.0057063

da Fontoura Budaszewski, R., von Messling, V., 2016. Morbillivirus experimental animal models: measles virus pathogenesis insights from canine distemper virus. Viruses 8, 274. https://doi.org/10.3390/v8100274

Dahl, 2018. First identification of feline morbillivirus in Swedish cat population (Advanced - A2E, MSc. thesis). Swedish University of Agricultural Sciences, Uppsala.

de Vries, R.D., Duprex, W.P., de Swart, R.L., 2015. Morbillivirus infections: an introduction. Viruses 7, 699–706. https://doi.org/10.3390/v7020699

Démoulins, T., Baron, M.-L., Kettaf, N., Abdallah, A., Sharif-Askari, E., Sékaly, R.-P., 2009. Poly (I:C) induced immune response in lymphoid tissues involves three sequential waves of type I IFN expression. Virology 386, 225–236. https://doi.org/10.1016/j.virol.2009.01.024

Devaux, P., Hodge, G., McChesney, M.B., Cattaneo, R., 2008. Attenuation of V- or C-defective measles viruses: Infection control by the inflammatory and interferon responses of rhesus Monkeys. J. Virol. 82, 5359–5367. https://doi.org/10.1128/JVI.00169-08

Fontana, J.M., Bankamp, B., Bellini, W.J., Rota, P.A., 2008. Regulation of interferon signaling by the C and V proteins from attenuated and wild-type strains of measles virus. Virology 374, 71–81. https://doi.org/10.1016/j.virol.2007.12.031

Fox, J.P., 1983. Herd immunity and measles. Rev. Infect. Dis. 5, 463–466. https://doi.org/10.1093/clinids/5.3.463

González-Navajas, J.M., Lee, J., David, M., Raz, E., 2012. Immunomodulatory functions of type I interferons. Nat. Rev. Immunol. 12, 125–135. https://doi.org/10.1038/nri3133

Horvath, C.M., 2004. Weapons of STAT destruction. Interferon evasion by paramyxovirus V proteins. Eur. J. Biochem. 271, 4621–4628. https://doi.org/10.1111/j.1432-1033.2004.04425.x

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M., Yanagi, Y., 2008. Measles virus circumvents the host interferon response by different actions of the C and V proteins. J. Virol. 82, 8296–8306. https://doi.org/10.1128/JVI.00108-08

Ohno, S., Ono, N., Takeda, M., Takeuchi, K., Yanagi, Y., 2004. Dissection of measles virus V protein in relation to its ability to block alpha/beta interferon signal transduction. J. Gen. Virol. 85, 2991–2999. https://doi.org/10.1099/vir.0.80308-0

Patterson, J.B., Thomas, D., Lewicki, H., Billeter, M.A., Oldstone, M.B., 2000. V and C proteins of measles virus function as virulence factors in vivo. Virology 267, 80–89. https://doi.org/10.1006/viro.1999.0118

Pfeffermann, K., Dörr, M., Zirkel, F., von Messling, V., 2018. Morbillivirus pathogenesis and virus–host interactions. Academic Press, pp. 75–98. https://doi.org/10.1016/bs.aivir.2017.12.003

Röthlisberger, A., Wiener, D., Schweizer, M., Peterhans, E., Zurbriggen, A., Plattet, P., 2010. Two domains of the V protein of virulent canine distemper virus selectively inhibit STAT1 and STAT2 nuclear import. J. Virol. 84, 6328–6343. https://doi.org/10.1128/JVI.01878-09

Sanz Bernardo, B., Goodbourn, S., Baron, M.D., 2017. Control of the induction of type I interferon by Peste des petits ruminants virus. PLOS ONE 12(5): e0177300. https://doi.org/10.1371/journal.pone.0177300

Shaffer, J.A., Bellini, W.J., Rota, P.A., 2003. The C protein of measles virus inhibits the type I interferon response. Virology 315, 389–397. https://doi.org/10.1016/S0042-6822(03)00537-3

Sharp, C.R., Nambulli, S., Acciardo, A.S., Rennick, L.J., Drexler, J.F., Rima, B.K., Williams, T., Duprex, W.P., 2016. Chronic infection of domestic cats with feline morbillivirus, United States. Emerg. Infect. Dis. 22, 760–762. https://doi.org/10.3201/eid2204.151921

Sparrer, K.M.J., Pfaller, C.K., Conzelmann, K.-K., 2012. Measles virus C protein interferes with beta interferon transcription in the nucleus. J. Virol. 86, 796–805. https://doi.org/10.1128/JVI.05899-11

Su, J., Dou, Y., You, Y., Cai, X., 2015. Application of minigenome technology in virology research of the Paramyxoviridae family. J. Microbiol. Immunol. Infect. 48, 123–129. https://doi.org/10.1016/j.jmii.2014.02.008

Svitek, N., Gerhauser, I., Goncalves, C., Grabski, E., Döring, M., Kalinke, U., Anderson, D.E., Cattaneo, R., Messling, V. von, 2014. Morbillivirus control of the interferon response: Relevance of STAT2 and mda5 but not STAT1 for canine distemper virus virulence in ferrets. J. Virol. 88, 2941–2950. https://doi.org/10.1128/JVI.03076-13

Swiss Institute of Bioinformatics, 2018. Morbillivirus. ViralZone.

Woo, P.C.Y., Lau, S.K.P., Wong, B.H.L., Fan, R.Y.Y., Wong, A.Y.P., Zhang, A.J.X., Wu, Y., Choi, G.K.Y., Li, K.S.M., Hui, J., Wang, M., Zheng, B.-J., Chan, K.H., Yuen, K.-Y., 2012. Feline morbillivirus, a previously undescribed paramyxovirus associated with tubulointerstitial nephritis in domestic cats. Proc. Natl. Acad. Sci. U. S. A. 109, 5435–40. https://doi.org/10.1073/pnas.1119972109

Yokota, S., Okabayashi, T., Fujii, N., 2011. Measles virus C protein suppresses gamma-activated factor formation and virus-induced cell growth arrest. Virology 414, 74–82. https://doi.org/10.1016/j.virol.2011.03.010

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Appendix 1 – The details of V and C plasmid vectors

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---------------------------------------------
The details of FmoPV, V and C plasmid vectors
---------------------------------------------

Gene name: FmoPV - KR014147.1:1812-2324 Feline morbillivirus strain US1,
CDS - V gene

Vector name: pCMV-3Tag-1a,
Cloning site: EcoRI/HindIII,


Length: 843 bp,
Additional 5' sequence: GAATTC,
Additional 3' sequence: AAGCTT,
Start with : GAATTC,

Sequence:
ATGTCCTCTCACCAAATCCAACAAGTCAAACATGGCCTTGAATCTTTACAAGAGATCAAAAACAACCCTCCGTCTTCCCAAGATGTCAATCTTGC
CAGGGAGATTTACGAATCCATTAGACAAGCAGGAACATCTTCAGTGCAAGGAGGAGCCATTGCGAGAGATAATATTACGTCAGGGGGTAACAATGACTCA
ATGTATAGCCAAGGACCAAGTCCTCCTATTTCAAGTGTTAACAAGAATATCGAAGGACCTACTGGATTCGATCATTCAGGACTATGGGATCCAGAGGGTA
ACCTCTGCATGCTATTCGAAAGCGATGATGATGAAAACCATTATTCAGAGATTAATGGCCGGTCTTCCACTATCGAAGGACTGGATGAACAGGATAATGA
GGACTCAATTATTAAACAACCAGGAAATCAGTGTACTGAGGGAGTGTCTAAGACTGATTCATCTCTTAGTTCCCAGGAAACTACACTATCTGTTGGGGGA
TCTGATATACCTGGGACAGGAATATCAACCTGTGCCTCTTTGGATATAACTGTAAATGAACTCGAAGATGCAACTGTAAGAAATAGCAACAATATGAAAG
GGAACTGGCCAATTCCTAAATTACTCGTTAAGCCGCCACCTAGGGTAAAATCAAGTGTTGATCACAGTAATCCATTAAAAGGGGGCCACAGAAGGGAAAT
TAGCCTCACCTGGGATGGAGACTACATTATTCGAGAAGAGTGGTGCAACCCTATCTGTACACCCATATACTCAACCTGCAAGCGACTACAATGTAGGTGC
AAGCAATGTCCATCAACCTGCCCTAAATGTGAATAA,
End with : AAGCTT,


Gene name: FmoPV - KR014147.1:1812-2324 Feline morbillivirus strain US1,
CDS - V gene

Vector name: pCMV-3Tag-1a,
Cloning site: EcoRI/HindIII,

Length: 525 bp,
Additional 5' sequence: GAATTC,
Additional 3' sequence: AAGCTT,
Start with : GAATTC,

Sequence:
ATGGCCTTGAATCTTTACAAGAGATCAAAAACAACCCTCCGTCTTCCCAAGATGTCAATCTTGCCAGGGAGATTTACGAATCCATTAGACAAGCA
GGAACATCTTCAGTGCAAGGAGGAGCCATTGCGAGAGATAATATTACGTCAGGGGGTAACAATGACTCAATGTATAGCCAAGGACCAAGTCCTCCTATTT
CAAGTGTTAACAAGAATATCGAAGGACCTACTGGATTCGATCATTCAGGACTATGGGATCCAGAGGGTAACCTCTGCATGCTATTCGAAAGCGATGATGA
TGAAAACCATTATTCAGAGATTAATGGCCGGTCTTCCACTATCGAAGGACTGGATGAACAGGATAATGAGGACTCAATTATTAAACAACCAGGAAATCAG
TGTACTGAGGGAGTGTCTAAGACTGATTCATCTCTTAGTTCCCAGGAAACTACACTATCTGTTGGGGGATCTGATATACCTGGGACAGGAATATCAACCT
GTGCCTCTTTGGATATAA,
End with : AAGCTT,
```

Figure 1: Details of V and C plasmid vectors of FmoPV. As restriction sites, EcoRI at the 5'end and HindIII at the 3' were used for both cDNAs

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---------------------------------------------
The details of CDV, V and C plasmid vectors
---------------------------------------------

Gene name: KF914669.1:1823-2347 Canine distemper virus isolate CDV2784/2013,
CDS - V gene

Vector name: pCMV-3Tag-1a,
Cloning site: EcoRI/HindIII,


Length: 912 bp,
Additional 5' sequence: GAATTC,
Additional 3' sequence: AAGCTT,
Start with : GAATTC,
Sequence:
ATGGCAGAGGAGCAGGCCTATCATGTCAGTAAAGGGCTGGAATGCCTCAAAGCCCTCAGAGAGAATCCTCCTGACATTGAGGAGATTCAAGAGGT
CAGCAGCATCAGAGACCAAACCCGCAACCCAGGCCAAGAGAATGGAACCGCAAGCATGCAAGAAGAGGAGGTCTCTCAGGATCTCGATGAATCACACGAG
CCAGCAAAAGGATCAAAATATGTCGGCCATGTACTCCAAAACAATCCGGGATGTGGAGAGAGCAACACTGCGCTTGTGGAGGCAGAGCAACCCGCTAAAG
ATGACATCCAACCAGGACCTGGAGTACGATGTTATCATGTTTATGATCACAGCGGTGAAGAGGTTAAGGGAATCGAAGATGCTGACAGTCTCGTGGTACC
TGCAGGCGCTGTCAGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAGCACTGAGGATTCTGGCGAAGATTATTCCGAAGGAAATGCTTCA
TCTAACTGGGGATATTCTTTCGGCCTTAAACCAGACAGAGCAGCTGATGTAAGTATGCTGATGGAAGAGGAATTGAGTGCTCTGCTCAGGACAAGCAGAA
ATGTAGGGATTCAGAAAAGGGATGGGAAAACTCTGCAGTTCCCACACAATCCCGAAGGTAAGACAGGGGATCCGGAGTGTGGATCCATTAAAAAGGGGCA
CAGGAGAGAAGTCAGCCTCACATGGAATGGGGATAGTTGCTGGATCGACAAATGGTGCAACCCAATCTGCACCCAAGTCAACTGGGGGATCATCAGGGCC
AAGTGTGTCTGTGGAGAATGTCCGCCAACCTGCAATGAGTGCAGAGATGACCCAGAAATGCAAACCCGAGTCTGGTACGCAACCCCCTCCCAGGACCTCA
AATGA,
End with : AAGCTT,


Gene name: KF914669.1:1823-2347 Canine distemper virus isolate CDV2784/2013,
CDS - C gene

Vector name: pCMV-3Tag-1a,
Cloning site: EcoRI/HindIII,

Length: 537 bp,
Additional 5' sequence: GAATTC,
Additional 3' sequence: AAGCTT,
Start with : GAATTC,

Sequence:
ATGTCAGTAAAGGGCTGGAATGCCTCAAAGCCCTCAGAGAGAATCCTCCTGACATTGAGGAGATTCAAGAGGTCAGCAGCATCAGAGACCAAACC
CGCAACCCAGGCCAAGAGAATGGAACCGCAAGCATGCAAGAAGAGGAGGTCTCTCAGGATCTCGATGAATCACACGAGCCAGCAAAAGGATCAAAATATG
TCGGCCATGTACTCCAAAACAATCCGGGATGTGGAGAGAGCAACACTGCGCTTGTGGAGGCAGAGCAACCCGCTAAAGATGACATCCAACCAGGACCTGG
AGTACGATGTTATCATGTTTATGATCACAGCGGTGAAGAGGTTAAGGGAATCGAAGATGCTGACAGTCTCGTGGTACCTGCAGGCGCTGTCAGTAATCGA
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GCCTTAAACCAGACAGAGCAGCTGATGTAA,
End with : AAGCTT,
```

Figure 2: Details of V and C plasmid vectors of CDV. As restriction sites, EcoRI at the 5'end and HindIII at the 3' were used for both cDNAs

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--------------------------------------------
The details of MeV, V and C plasmid vectors
--------------------------------------------

Gene name: MeV - DQ227319.1:1829-2389 Measles virus isolate 97-45881,
CDS - V gene

Vector name: pCMV-3Tag-1a,
Cloning site: BamHI/EcoRI
Length: 912 bp,
Additional 5' sequence: GGATCC,
Additional 3' sequence: GAATTC,
Start with : GGATCC,

Sequence:
ATGGCAGAAGAGCAGGCACGCCATGTCAAAAACGGACTGGAATGCATCCGGGCTCTCAAGGCCGAGCCCATCGGCTCACTGGCCGTCGAGGAAGC
CATGGCAGCATGGTCAGAAATATCAGACAACCCAGGACAGGACCGAGCCACCTGCAAGGAAGAGAAGGCAAGCAGTTCGGGTCTCAGCAAACCATGCCTC
TCAGCAATTGGATCAACTGAAGGCGGTGCACCTCGCATCCGCGGTCAGGGATCTGGAGAGAGCGATGACGACGCTGAAACTTTGGGAATCCCCTCAAGAA
ATCTCCAGGCATCAAGCACTGGGTTACAGTGTTATCATGTTTATGATCACAGCGGTGAAGCGGTTAAGGGAATCCAAGATGCTGACTCTATCATGGTTCA
ATCAGGCCTTGATGGTGATAGCACCCTCTCGGGAGGAGACGATGAATCTGAAAACAGCGATGTGGATATTGGCGAACCTGATACCGAGGGATATGCTATC
ACTGACCGGGGATCTGCTCCCATCTCTGTGGGGTTCAGGGCTTCTGATGTTGAAACTGCAGAAGGAGGGGAGATCCATGAGCTCCTGAGACTCCAATCCA
GAGGCAACAACTTTCCGAAGCTTGGGAAAACTCTCAATGTTCCTCCGCCCCCGAACCCCGGTAGGGCCAGCGCTTCCGAGACACCCATTAAAAAGGGGCA
CAGACGCGAGATTAGCCTCATTTGGAACGGAGATCGCGTCTTTATTGACAGGTGGTGCAACCCAATGTGCTCGAAAGTCACCCTCGGAACCATCAGGGCT
AGGTGCACATGCGGGGAATGTCCCCGAGTGTGTGAGCAATGCCGCACTGATACAGGAGTGGACACCCGAATCTGGTACCACAACCTCCCCGAGATCCCAG
AATAA,
End with : GAATTC,

Gene name: MeV_C_2005,

Vector name: pCMV-3Tag-1a,
Cloning site: BamHI/EcoRI

Length: 573 bp,
Additional 5' sequence: GAATTC,
Additional 3' sequence: AAGCTT,
Start with : GAATTC,

Sequence:
ATGTCAAAAACGGACTGGAATGCATCCGGGCTCTCAAGGCCGAGCCCATCGGCTCACTGGCCGTCGAGGAAGCCATGGCAGCATGGTCAGAAATA
TCAGACAACCCAGGACAGGACCGAGCCACCTGCAAGGAAGAGAAGGCAAGCAGTTCGGGTCTCAGCAAACCATGCCTCTCAGCAATTGGATCAACTGAAG
GCGGTGCACCTCGCATCCGCGGTCAGGGATCTGGAGAGAGCGATGACGACGCTGAAACTTTGGGAATCCCCTCAAGAAATCTCCAGGCATCAAGCACTGG
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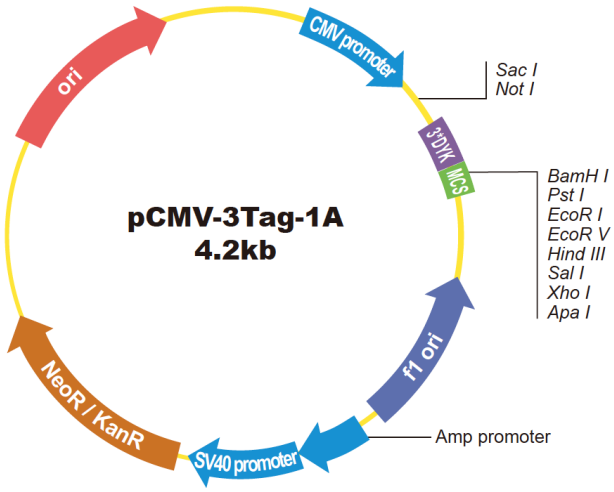
ACCCTCTCGGGAGGAGACGATGAATCTGAAAACAGCGATGTGGATATTGGCGAACCTGATACCGAGGGATATGCTATCACTGACCGGGGATCTGCTCCCA
TCTCTGTGGGGTTCAGGGCTTCTGATGTTGAAACTGCAGAAGGAGGGGAGATCCATGAGCTCCTGA,
End with : AAGCTT,
```

Figure 3: Details of V and C plasmid vectors of MeV. As restriction sites, BamHI at the 5'end and EcoRI at the 3' were used for both cDNAs



3× DYK tag

--- G AGC TCC ACC GCG GTG GCG GCC GCC ACC ATG GAT TAC AAG GAT GAC GAC GAT AAG GAC TAT AAG GAC GAT GAT
Sac I *Not* I M D Y K D D D D K D Y K D D D

3× DYK tag

GAC AAG GAC TAC AAA GAT GAT GAC GAT AAA GCC CGG GCG GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT
D K D Y K D D D D K *Bam*H I *Pst* I *Eco*R I *Eco*R V *Hind* III

ATC GAT ACC GTC GAC CTC GAG GGG GGG CCC ---
Sal I *Xho* I *Apa* I

Figure 4: Vector map of pCMV-3Tag-1A

Appendix 2 – Experimental setup

Transfection:

Table 1: Experiment setup – Per well (containing 0.5 ml growth media with cells) according to the concentration of plasmids

	pcDNA 1000 ng/ml	***V or C plasmid 250 ng/ml***	***V or C plasmid 500 ng/ml***	***V or C plasmid 1000 ng/ml***
Plasmids &Reagents	*Microliters*	*Microliters*	*Microliters*	*Microliters*
OPTI	*50*	*50*	*50*	*50*
ISRE (200 ng/ml)	*0.5*	*0.5*	*0.5*	*0.5*
Renilla (2 ng/ml)	*0.5*	*0.5*	*0.5*	*0.5*
pcDNA (250 ng/ml)	***2***	***1.5***	***1***	
V or C Protein (250 ng/ml)		***0.5***	***1***	***2***
Trans IT-LTI	*1.8*	*1.8*	*1.8*	*1.8*
Total per well	***54.8***	***54.8***	***54.8***	***54.8***