PDZ Binding Motif of NS1 Proteins of Influenza A Viruses:
A Virulent Factor in the Expression of Interferon-β?

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ABSTRACT

Background:
The PDZ domain is a peptide sequence of 80-90 amino acids and can be found in e.g. bacteria, animals and plants. These domains are commonly part of the cytoplasmic and membrane adapter proteins and its function are important in protein-protein interactions. The NS1 proteins of influenza A viruses play an important role in inhibiting the IFN-β production in many ways. In the C-terminus of the NS1 protein, a peptide sequence of four amino acids had been demonstrated to bind to the PDZ domain termed as PDZ binding motif (PBM).

Objective:
The aim of this study is to determine whether the PBM sequence of the NS1 protein of influenza A virus plays a key roll in the expression of interferon-β.

Methods:
The open reading frame of the NS1 protein was amplified and cloned into expressing vector and transfected into A549 cells along with a reporter plasmid containing ISRE promoter, driving expression of firefly luciferase. Dual luciferase reporter assay was performed to measure luciferase activity which represented expression of IFN-β. The assay was performed only once and unfortunately the result can not be trusted since the negative control showed positive value. Therefore, to understand the interaction between the PBM sequence of NS1 proteins and the production of IFN-β, further experiments are needed.

Keywords: H5N1, PBM, avian influenza viruses, non-structural protein 1, IFN-β production.
ABBREVIATIONS

HA - hemagglutinin
ISRE - interferon-sensitive response element
NA - neuraminidase
NS - non-structural
NS1 - non-structural protein 1
PDZ - acronym representing PSD95/DLG/ZO-1
PBM - PDZ domain binding motif
rDNA - recombinant DNA molecule
INTRODUCTION

In 1918, the deadly pandemic disease “Spanish flu” was caused by influenza A viruses and 50-100 millions of people globally lost their lives (Johnson & Mueller 2002). Many of those who were afflicted either died from suffocation that is a result from pulmonary oedema or from bacterial infections. Another historic incident was in Hong Kong 1997 when the viruses, for the first time documented, succeeded in infecting humans and killed six people.

Influenza is an infectious airborne disease caused by RNA viruses. There are three influenza genera, influenza A, B and C, which belong to the Orthomyxoviridae family. Influenza A and B viruses are of more general interest because they may cause local outbreaks (epidemic) among humans with symptoms as fever, malaise, headache and pain in the body muscles (myalgias) while influenza C viruses cause common cold. However, only influenza A viruses are able to spread the disease all around the world (pandemic) and are still dangerous for humans.

Influenza A viruses have wild waterfowls as their natural hosts, spreading the disease among birds and therefore named avian influenza (bird flu). In domestic poultry population there are clinical signs as decreasing egg production, oedema, diarrhea and more. This result in severe financial damages for the poultry industries worldwide as the breeders have to destroy the infected herds. Furthermore, subtypes of avian influenza viruses are divided in low pathogenic avian influenza viruses, which cause mild diseases, and high pathogenic avian influenza viruses that may kill up to 100% of the poultry herd in a short amount of time.

The subtypes of the viruses are based on the two surface proteins, HA (hemagglutinin) and NA (neuraminidase). There are 16 subtypes of HA proteins (H1-H16) and 9 subtypes of NA proteins (N1-N9) found in wild aquatic birds, so theoretically there are 144 (16x9) combinations of subtypes ranging from H1N1 to H16N9. Recently genetic material from a new subtype (H17) was discovered in
Central American Bats (Tong et al. 2012).

Epithelial cells in the upper respiratory tract are protected by a layer of mucus secretion rich in NANA (N-acetyl neuraminic acid), i.e. sialic acid. The HA proteins bind specific to the NANA receptors which give the viruses a chance to adhere to the mucus. The NA proteins also bind to the NANA receptors but instead it will enzymatically break down these receptors, making it possible for the viruses to reach and infect the cells. Furthermore, by cleaving NANA receptors from the infected cell, NA proteins also participate in budding of new virions.

RNA viruses have strong disposition for mutations and thus increase the chances of evading the immune system. The canyon-like binding part of HA proteins of influenza A viruses consist of one invariant zone in the middle surrounded by protruding and highly mutated zones (Wiley et al. 1987). Due to the modifications in the protruding zones, the antibodies can not bind to the HA proteins which result in infection and annual outbreaks of influenza A viruses known as antigenic drift.

Seasonal influenza viruses only bind to the α(2,6) linkage, the major type of NANA receptors commonly found in the upper respiratory tract in, inter alia, humans. Ducks, on the other hand, express more of the other type which are of α(2,3) linkage and the avian influenza A viruses preferably bind to these kind of NANA receptors (Connor et al. 1994). Moreover, various tissues of ducks and chickens also express both α(2,3) and α(2,6) linkage and likewise the epithelial cells of the lower respiratory tract in pigs. This may result in co-infections by both human influenza A viruses and avian influenza A viruses (Trebbien et al. 2011; Kuchipudi et al. 2009). Since the genome of influenza A viruses consist of eight segments, viruses of various strains may interchange the genome parts and give rise to new strains. This process of gene reassortment is known as antigen shift.

The influenza A virions have several shapes, commonly, spherical or filamentous with a diameter of 80-120nm. The virions are enveloped by a lipid bilayer with two crucial glycoproteins (HA and NA) attached to it. Additionally, the M2 (matrix protein 2) which is a transversal ion-channel can be found on the surface of the
envelope. The M1 (matrix protein 1) lines up along the inside of the envelope. The genetic materials lie within the envelope which consist of eight helical nucleocapsid segments. Each one of these segments contains a negative-sense ssRNA with NPs (nucleoprotein) bound to it as a shelter. Moreover, three subunits of the RNA-dependent RNA polymerase, PB1 (polymerase basic protein 1), PB2 (polymerase basic protein 2) and PA (polymerase acidic protein), are part of the nucleocapsid segment which are also termed as vRNP (viral ribonucleoprotein).

These eight vRNPs differ in size and are arranged from the largest to the smallest: PB2, PB1, PA, HA, NP, NA, M (matrix) and NS (non-structural). The following segments PB2, PA, HA, NP and NA encode only for one protein respectively while the M and NS segments are able to splice the RNA and therefore encode for additional proteins. The PB1 gene segment encodes for three proteins through an alternative reading frame: PB1, PB1-F2 and N40 and the M gene segment encodes for two proteins: M1 and M2. The eighth and smallest segment, NS, encodes for two proteins: NS1 (non-structural protein 1) and NEP (nuclear export protein).

The NS gene of influenza A viruses is an 890bp RNA template resulting in the translation of a 230 amino acids long NS1 protein. Furthermore, the mRNA may as well undergo splicing resulting in the NEP protein. Dundon & Capua (2009) reviewed that the strain-specific length of the NS1 proteins are not absolute but vary from 202 to 237 amino acids. The NS1 proteins further exist in two alleles, A and B (Suarez & Perdue 1998). It has been demonstrated that allele A of the NS1 proteins inhibit the transcription process of the IFN-β gene more efficiently than allele B (Zohari et al. 2010).

The NS1 protein consists of two functional parts, an N-terminal RBD (dsRNA binding domain) (residues 1-73 amino acids) (Chien et al. 2009; Liu et al. 1997) and a C-terminal ED (effector domain) (residues 74-230 amino acids) (Bornholdt & Prasad 2006; Xia et al. 2009). These two domains exist in a symmetric homodimer by dimerization of two monomers of two NS1 proteins. The dimerization is important for the RBD to bind to the dsRNA (Wang et al. 1999). Moreover, it has
been observed that the crystal structure of the functional parts of one NS1 protein may dimerize with other NS1 proteins, forming a long NS1 complex with alternate RBD and ED dimers (Bornholdt & Prasad 2008).

Previous studies show that the NS1 protein bears multifunctional qualities contributing to the viral replication. The two domains of the NS1 protein bind and interact with different host-cell proteins and thus affecting various signalling pathways resulting in, inter alia, inhibition of the immune response or enhancement of the viral translation (Hale *et al.* 2008). For example, the RBD inhibits activation of PKR (protein kinase R) and subsequently decrease phosphorylation of eIF2α (eukaryotic initiation factor 2 α) (Lu *et al.* 1995). The main cause of phosphorylation of eIF2α is to block the cellular translation process in infected cells to prevent further viral protein synthesis. Furthermore, the ED is important for stabilizing the RBD (Wang *et al.* 2002).

Another important role of the NS1 protein is to inhibit production of IFN (interferon) α and β which are expressed by virus infected cells to put the non infected cells into an antiviral state. The IFN-β production is limited by the NS1 protein by pre-transcriptional process in the cytoplasm and/or by post-transcriptional process in the nucleus (Hale *et al.* 2008). A study demonstrated that in the pre-transcriptional process, the NS1 protein forms a complex with the RIG-1 (retinoic acid–inducible gene I) and hence limits the IFN-β production. RIG-1 is an intracellular receptor which is required to induce IFN-β by binding to dsRNA (Guo *et al.* 2007). In the post-transcriptional process the NS1 proteins form a complex with the CPSF30 (human 30kDa subunit of cleavage and polyadenylation specificity factor) which prevents cleavage and polyadenylation of the host-cell's pre-mRNA. This will result in halting of the gene expression (Nemeroff *et al.* 1998).

The PDZ (acronym of PSD95/DLG/ZO-1) domain is a peptide sequence of approximately 90 amino acids and widespread among various organisms such as bacteria, yeast, plants and animals. In multicellular organisms, a single protein may express multiple copies of the PDZ sequence which is commonly found in
cytoplasmic and membrane adapter proteins. The domain is important in protein-protein interactions, participating in formation of protein complexes. These complexes of proteins are of importance for, inter alia, signalling pathways and cell junctions (Javier & Rice 2011; Yu et al. 2011; Fanning & Anderson 1999). The PBM (PDZ domain binding motif) sequence is residues of four amino acids at the C-terminus of the NS1 protein and has been identified as ESEV in 78% of the avian isolates by Obenauer et al. in 2006. The PBM sequence of the NS1 proteins was revealed as a virulent determinant (Jackson et al. 2008) and a study demonstrated that the PBM sequence ESEV disrupts tight junctions in cells (Golebiewski et al. 2011).

To reveal additional pathogenic effects of the PBM sequence of NS1 proteins of influenza A viruses, further research is required. Therefore, the aim of this study was to observe the role of the PBM sequence in the expression of the IFN-β.

**MATERIALS AND METHODS**

**Samples**

In 2006, during the outbreak of highly pathogenic avian influenza H5N1 in wild birds in Europe tissue samples were taken from a mink in Sweden and a swan in Bosnia. Before RNA extraction, the tissue samples were prepared in a BSL3 (biosafety level 3) laboratory of the National Veterinary Institute in Sweden to inactivate the virions by Trizol. The sample isolated from mink was named A/Mink/Sweden/2006/H5N1 and the sample isolated from swan was named A/Swan/Bosnia/2006/H5N1. There was permission from Swedish Work Environment Authority for working with plasmids because in this study, microorganisms were genetic manipulated.

**RNA Extraction**

For each sample this step was performed as followed: To inactivate the viruses,
500µL of TRIzol was transferred into 200µL sample.

At the “Phase Separation” - 250µL chloroform was added and mixed by turning up and down several times, followed by 5 minutes incubation at room temperature. The mixing and the incubation steps were repeated and subsequently centrifuged at 16000xg at 4°C for 10 minutes using a refrigerated centrifuge. This resulted in three distinct phases within the tube, the clear upper phase containing the wanted RNA, the milky intermediate phase with DNA and the red/pink phase at the bottom of the tube with lipids, proteins and cell rests.

At the “RNA Precipitation” step, only the clear upper phase was cautiously transferred over to a sterile tube. Thereafter, 500µL of 2-propanol was added followed by 10 minutes incubation at room temperature to precipitate the RNA. The tube was centrifuged at 16000xg at 4°C for 10 minutes.

In the following step, “RNA Wash and Resuspension”, the supernatant was discarded and to resuspend the pellet, 500µL 75% (v/v) ethanol was added into the tube and the content was mixed up for a few seconds using a vortex-mixer. The tube was centrifuged at 11000xg at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 500µL 75% (v/v) ethanol. The tube was briefly vortex-mixed and centrifuged at 11000xg at 4°C for 5 minutes. The supernatant was discarded and the tube was air dried for 20 minutes. The pellet was thereafter dissolved in 20µL RNase-free water, then vortex-mixed and finally the tube was briefly spun down. If the sample was not used immediately it was stored at -20°C.

**Reverse Transcription quantitative PCR**

RT-qPCR was performed with OneStep RT-PCR kit (Qiagen, Germany) to control the “RNA Extraction” step, e.g. if any contamination had occurred and also to screen for the M gene which is highly conserved for influenza A viruses (Furuse et al. 2009). For each sample the master mix was prepared as followed: 7.1µL of RNase-free water was transferred into a sterile tube. The following reagents were then added: 3µL of 5XRT PCR, 0.75µL MgCl₂ (25mM), 0.6µL dNTP (10mM), 0.3µL of forward primer (5’-AGA TGA GTC TTC TAA CCG AGG TCG-3’) (10µM), 0.3µL of
reverse primer (5'-TGC AAA AAC ATC TTC AAG TCT CTG-3') (10µM) and 0.3µL of the probe (5'-FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA-3') (10µM). Additionally, 0.06µL of RNA guard and 0.6µL of enzyme mix completed the master mix and was spun down quickly. Thereafter, 13µL of the master mix was transferred into a PCR tube placed on a chilled tube holder, and then 2µL of the RNA sample from the “RNA Extraction” step was added, giving a total volume of 15µL in the PCR tube.

The RT-qPCR was performed by Rotor-Gene RG3000 (Corbett Research, UK). Briefly, in the first two steps, the RNA was transcribed into cDNA by the reverse transcriptase and then separated into ssDNA. This was followed by a thermal cycle to amplify the cDNA and FAM (fluorescein amidite) fluoresceins was measured at each cycle. The thermal profile was as followed: Reverse transcriptase transcribed RNA into cDNA at 50ºC for 30 minutes. The cDNA was initially separated at 95ºC for 15 minutes. The cDNA denatured at 95ºC for 10 seconds. Annealing and elongation at 58ºC for 20 seconds and FAM fluoresceins was measured, using ROX (carboxy-x-rhodamine) and FAM filters. Step 3 to 4, was repeated 40 times. The process ended at 50ºC for 1 minute and the amplicon was kept cold at 4ºC until use.

**Reverse Transcription PCR**

Those RNA samples which were positive for influenza A at the “Reverse Transcription quantitative PCR” step were run by RT-PCR to transcribe the RNA into cDNA. Random RNA dependent primer (Life Technologies, US) was used to randomly bind to the RNA. For each sample the master mix was prepared as followed: 2µL of RNase-free water was transferred into a sterile tube. Then 4µL 5XFS buffer, 2µL of random hexamers (50µM), 2µL dithiothretol (0.1M) to reduce disulphide bonds, 2µL MgCl₂ (25mM), 1µL dNTP (10mM), 1µL RNaseOUT (Life Technologies, US) and 1µL M-MLV (moloney-murine leukemia virus) Reverse Transcriptase (Life Technologies, US) were added.

Thereafter, 15µL of the master mix was transferred into a sterile PCR tube on ice. Additionally, 5µL of RNA sample was added, vortex-mixed and spun down briefly.
The thermal profile on the MiniCycler (MJ Research, US) was as followed: Annealing and elongation at 42ºC for 45 minutes. Enzyme inactivation at 60ºC for 15 minutes and finally, denaturation at 95ºC for 10 minutes. The PCR tubes containing cDNA (from now on only DNA) were kept at 4ºC until use.

**PCR of the NS Gene**

The NS gene was amplified by PCR and to facilitate the cloning of the NS gene, primers with recognition sites of endonucleases were used. The forward primer has the recognition site of the restriction enzyme KpnI (isolated from *Klebsiella pneumoniae OK8*) added at the 5'-end. The reverse primer on the other hand has the recognition site of XbaI (isolated from *Xanthomonas badrii*) added at the 5'-end.

For each sample, the master mix ingredients were transferred into a sterile PCR tube as followed: 30µL of RNase-free water, 5µL 10XPCR buffer, 3µL MgCl$_2$ (25mM), 1µL dNTP (10mM), 2µL of forward primer (NS1FKPN #116375 CyberGene, Sweden) (10µm/µL) and 2µL of reverse primer (NS1RXBA #116376 CyberGene, Sweden) (10µm/µL). The last ingredient, Taq polymerase Platinum was diluted 1:10 with RNase-free water before 2µL was transferred into the master mix tube. Furthermore, 5µL of the DNA sample from the “Reverse Transcription PCR” step was added into 45µL of the master mix in a sterile PCR tube on ice.

The PCR tubes were run by T3000 Thermocycler (Biometra, Germany) with following thermal profile: The lid heating temperature was set to 105ºC and the samples were preheated to 95ºC for 2 minutes. The DNA was denatured at 95ºC for 30 seconds then annealing at 52ºC for 1 minute and elongation at 72ºC for 1 minute. Steps 3 – 5 were repeated 35 times and finished at 72ºC for 7 minutes to stop all ongoing reactions and then the tubes were kept at 4ºC until use.

The amplicons were purified by Wizard SV Gel and PCR Clean-Up System kit (Promega, US) (from now on only Clean-Up System) according to the manufacturer's instructions and were run on agarose gel electrophoresis to visualize the DNA band pattern.

Additionally, the PCR step was repeated but with another primer pair, NS1-FLAG-
XBA1-F (#156063 CyberGene, Sweden) and NS1-FLAG-KPN-R (#156068 CyberGene, Sweden). The primers have sequences (flag) ensuring that the open reading frame of the NS1 protein will be assembled correctly into the pCMV-FLAG-MAT-Tag-1 expression vector. These primers will henceforth be termed as flag tagged primers. Subsequently, the amplicons were purified by Clean-Up System kit and checked by agarose gel electrophoresis.

**BigDye Terminator Cycle Sequencing**

The sequencing was performed to ensure that the amplicon holds the NS gene and also acted as an origin template as comparison to see if any modifications had occurred to the NS gene during the cloning steps.

The BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, US) was used in this step and all the samples and reagents were kept on ice all the time. For each sample two sterile PCR tubes with respective master mix were prepared – one tube with a master mix with only forward primer and the other with a master mix with only reverse primer. For each tube the master mix was prepared as followed: 8.2µL of RNase-free water was transferred into the tube. Thereafter, 2µL of the BigDye mix and 3µL of 5xBigDye Sequencing Buffer were added. Then 1.8µL of either forward or reverse primer (2µM) was transferred into the tube followed by 5µL of the purified amplicon. The tubes were run on a PCR machine with following thermal profile: Preheating at 96ºC for 1 minute. Denaturing at 95ºC for 15 seconds. Annealing at 50ºC for 10 seconds and elongation at 60ºC for 4 minutes, step 2 - 4 were repeated for 24 cycles and then kept at 4ºC, ready for DNA precipitation.

For each sample the precipitation of DNA was prepared as followed: 2µL of sodium acetate 3M with pH5.2 was mixed with 50µL 99% (v/v) ethanol and transferred into the sample-tube, then vortex-mixed briefly and incubated at room temperature for 20 minutes. After being centrifuged for 20 minutes at 19000xg the ethanol was removed without disturbing the pellet. 250µL of 75% (v/v) ethanol was added and vortex-mixed to suspend the pellet. The content was centrifuged for 20 minutes at 19000xg and then the ethanol was removed. The sample was left to air-
dry in darkness.

Before loading the amplicon in the Genetic Analyzer 3100 (Life Technologies, US), 13µL of formamide was added into each tube and mixed using the pipette to resuspend DNA. Formamide will stabilize the DNA and prevent secondary structures. The sequencing was performed according to the manufacturer's instructions.

**Cleavage and Ligation**

Two cleavage steps were performed to cleave the samples and the two vectors, pCMV-FLAG-MAT-Tag-1 expression vector (Sigma-Aldrich, US) and pcDNA3.1+ (Life Technologies, US). The first mentioned vector will henceforth termed as pCMV-flag. The pCMV-flag vector has a flag sequence integrated in the N-terminus resulting in a flag epitope fused with the expressed protein and can be used for detection by monoclonal antibodies targeting the flag epitope. The XbaI restriction enzymes were used in the first cleavage step and during these steps all the samples and reagents were kept on ice. The cleavage step was further processed in two separate ways, one to cleave the vectors and the other to cleave the samples.

The procedure of cleaving the vectors was as followed: For each vector, a mix was prepared by transferring 33.5µL of RNase-free water into a sterile PCR tube. Additionally, 5µL 10XNE Buffer 2 (BioLabs, US), 5µL 10XBSA (bovine serum albumin) (Purified BSA 100X (BioLabs, US) diluted 1:10) and 2.5µL of the restriction enzyme XbaI (#R0145L BioLabs, US) was added. Thereafter, 5µL of the vector was added into 50µL of the mix and then briefly vortex-mixed and spun down quickly.

The samples were cleaved by XbaI restriction enzyme as followed: For each sample a mix was prepared by transferring 12µL of RNase-free water into a sterile PCR tube. Subsequently, 5µL 10XNE Buffer2, 5µL 10XBSA and 2.5µL of XbaI enzyme were added. Thereafter, 45µL of the sample was added into 25µL of the mix, then vortex-mixed and spun down for a few seconds.

The samples and the vectors were then run by PCR with following thermal profile:
The XbaI enzymes cut the DNA at 37°C for 2 hours. The activity of the enzymes stopped at 65°C during 20 minutes and the samples were kept at 4°C until use. The samples and vectors were then controlled by agarose gel electrophoresis.

The samples and vectors, cleaved by XbaI, were purified using the Clean-Up System kit to remove all excessive products from the cleavage step. Thereafter, agarose gel electrophoresis was run to check the purity of the amplicons.

The second cleavage of the samples and the vectors was performed using restriction enzyme KpnI (#R0142M BioLabs, US). The procedure was as followed and all the samples and reagents were kept on ice: For each sample or vector, a mix was prepared by transferring 6µL 10XNE Buffer1 (BioLabs, US), 6µL 10XBSA and 3µL of the KpnI enzymes in a sterile PCR tube. Thereafter, 45µL of the sample was added into 15µL of the mix then vortex-mixed and centrifuged shortly.

The samples and vectors were then run by PCR with the same thermal profile as in the previous cleavage step, i.e., 37°C for 2 hours, 65°C for 20 minutes and 4°C until use. An agarose gel electrophoresis was performed to control the product.

The samples and vectors were purified to remove all the extra reagents from the KpnI cleavage step using Clean-Up System kit. Thereafter, agarose gel electrophoresis was performed to control the purity of the amplicons. Subsequently, the samples with the NS gene were ligated with the vectors forming an rDNA with a size of approximately 6Kb.

At the ligation step, two mix were prepared. One mix containing pCMV-flag and the other mix containing pcDNA3.1+. Those DNA samples with flag tagged primers were transferred into the mix containing pCMV-flag and those DNA samples without flag tagged primers were transferred into the mix containing pcDNA3.1+. For each sample the mix was prepared as followed and all the samples and reagents were kept on ice: 4µL of RNase-free water was transferred into a sterile 1.5mL eppendorf tube. Thereafter, 3µL of either pCMV-flag or pcDNA3.1+, 2µL 10xLigase Buffer and 1µL T4 DNA Ligase (#M0202L BioLabs, US) were added. Additionally, 10µL of the sample was transferred into 10µL of the mix, vortex-mixed and spun down quickly. Paraffin film was wrapped around the cap of the tube to prevent vaporization when
incubated overnight at 16ºC in water bath. The sample was analyzed by agarose gel electrophoresis to control the result. DNA fragments larger than 5Kb run slower in TBE (tris/borate/EDTA) buffer than in TAE (tris/acetate/EDTA) buffer. Furthermore, because the rDNA was in a relaxed circular form the mobility of the fragment will be slowed down. All these factors will result in undetectable bands on agarose gel electrophoresis if ligation was successful.

**Transformation of DH5α Cells**

At this step the cells will take up the vectors during heat shock, a process known as transformation. Thanks to the resistance gene incorporated into the vectors, only cells that had taken up the rDNA will survive when growing in medium with ampicilline. This allowed selection of cells containing rDNA.

All the samples and reagents were kept on ice and for each sample, the insertion of the rDNA into the cells was performed as followed: A sterile 1.5mL eppendorf tube was chilled on ice for at least 15 minutes before use. Additionally, 250µL of SOC (super optimal broth with catabolite repression) medium was transferred into a falcon tube and left until room temperature was attained. DH5α cells (*Escherichia coli*) were thawed on ice and then 30µL was transferred into the chilled eppendorf tube. Thereafter, 2.5µL of the sample was added into the chilled eppendorf tube and mixed gently using the pipette. The tube was incubated on ice for 30 minutes before heat shocked at 42ºC for 30 seconds to make the cell membrane permeable and immediately thereafter, put on ice for 2 minutes. The content was transferred into the falcon tube with SOC medium for recovery and incubated at 37ºC for at least 1 hour shaking at 200-220rev/min.

An LB (lysogeny broth) agar plate was incubated at 37ºC for 15 minutes before use. Subsequently, 15µL of the suspension from the falcon tube was spread on LB agar plate and incubated in a container at 37ºC for about 14 hours. The lid was faced down to avoid condense in the agar medium. The falcon tube and the sample plate were kept at 4ºC until use.

The procedure of making LB plates was as followed: 5g of tryptonol, 2.5g of yeast
extract, 5g of NaCl and 7.5g of bactoagar were added into 500mL of distilled water. Subsequently, 500µL of ampicilline (5mg/mL) was added and 10mL of the medium was poured into a sterile plate and was left to solidify. The plates were kept in 4ºC until use.

**Master Plate**

A master plate was needed to keep all the bacterial colonies organised. All the samples and reagents were kept on ice all the time and for each sample plate from the transformation step, a master plate of LB medium was prepared as followed: 50 squares, numbered 1-50, were marked at the bottom of the master plate. One colony from the sample plate was picked up, without touching other colonies to avoid contamination, using an 1µL inoculating loop and stroke on square number 1. This moment was repeated until desired amount colonies have been picked up and stroken on respective squares. The master plate was incubated at 37ºC overnight with the lid faced down.

**Screening of Recombinant Colonies**

To know which cells that contained rDNA, a colony PCR with promoter primers was performed to screen for these recombinant colonies. Vectors usually have binding sites for these primers. By binding to these priming sites and amplifying the insert within, one could say that the insert is vector specific.

At this step, all samples and reagents were kept on ice all the time and for each colony which contained the pcDNA3.1+ vector, a master mix was prepared as followed: 17.5µL of RNase-free water was transferred into a sterile tube. Thereafter, following reagents were added: 2.5µL 10XPCR Buffer, 1.5µL MgCl₂ (25mM), 0.5µL dNTP (10mM), 0.5µL of T7 promoter primer (10µm), 0.5µL of the Sp6 promoter primer (10µm) and 2µL Taq Polymerase Platinum (diluted 1:10).

For each colony that contained the pCMV-flag vector, a master mix was prepared exactly as in the pcDNA3.1+ master mix but with different primer pair. Instead of T7 and Sp6 promoter primers, N-CMV-30 (Sigma-Aldrich, US) promoter primer pair
were used instead.

Then 25µL of the master mix was transferred into a sterile PCR tube. Thereafter, with a pipette tip, a colony was picked from a square on the master plate and transferred into the master mix. The suspension was mixed using the pipette and centrifuged briefly.

The sample was amplified by PCR with following thermal profile: 95ºC for 5 minutes to lysis the bacteria. Denaturation of DNA at 95ºC for 30 seconds then 50ºC for 30 seconds to hybridize primers and DNA. Subsequently, elongation at 72ºC for 2 minutes. Step 2 – 4 was repeated 34 times. The PCR finished at 72ºC for 7 minutes to make sure that the elongation of the product was complete and the tube was kept at 4ºC until use. Agarose gel electrophoresis was performed to control the amplicon. Clean-Up System kit was used to purify the amplicon containing rDNA.

Colonies with the required band were picked from the master plate and grown in LB medium to multiply. Each positive sample from the screening of recombinant colony step was prepared as followed: 3mL of LB medium with 1% NaCl was transferred into a new falcon tube. Subsequently, 3µL of ampicilline (50mg/mL) was added and then, with a pipette tip, one colony from the master plate was picked and the tip was kept within the tube. The sample was incubated shaking at 200-220rev/min overnight at 37ºC with the cap loosely tighten.

In the next step the rDNA was isolated and purified using the Wizard Plus SV Minipreps DNA Purification System + Vacuum Adapters kit (Promega, US). The process was performed according to the manufacturer's instructions and subsequently, the concentration of the rDNA was measured. The samples with the purified rDNA were kept on ice if used immediately, otherwise at -20ºC (at -70ºC for a longer period).

Transfection of A549 Cells

Transfection is the process where foreign DNA is introduced in eukaryotic cells while transformation is the process where foreign DNA is introduced in prokaryotic cells. Lipofectamine 2000 (Life Technologies, US) is a reagent for transfection of
mammalian cells. Basically, the reagent contains cationic lipids entrapping the anionic DNA thus forming a liposome/nucleic acid complex. The complex enters the cell by fusing with the cell membrane or by endocytosis to deliver DNA into the cell.

Before transfection, the A549 cells (human lung adenocarcinoma epithelial cell line) were cultured in 24-well plate incubated in F-DMEM medium (SVA, Sweden) at 37°C with 5% CO₂ for 24 hours to attain 80% confluence. Each sample was prepared in duplicates for the transfection. For each well the medium was changed as followed: The F-DMEM medium was incubated in waterbath at 37°C for 50 minutes. Then, the old medium in the transfection plate was removed and 500µL of F-DMEM medium containing 10% FBS (fetal bovine serum) was gently added into the wells, with the tip rested against the wall of the well. The plate was incubated at 37°C for 1 hour. The transfection was then performed according to the manufacturer's protocol (for 24-well plate), with following modification, 500ng of plasmid DNA was used instead of 100-200ng. The rDNA with the NS gene was co-transfected along with pISRE-Luc (Clontech, US) reporter vector expressing luciferase and pTA-Luc (Clontech, US) as an internal control to eliminate background activity. Empty pcDNA3.1+ vector was used as negative control. The plate was incubated at 37°C for 18-24 hours.

**Poly I:C Stimulation**

Poly(I:C) is a synthetic analogue of viral dsRNA and also ligand to anti viral sensory receptors in the cytoplasm such as TLR3 (toll-like receptor 3), RIG-1 and MDA5 (melanoma differentiation-associated gene 5). When the sensory receptors bind to the poly (I:C), cascades in different pathways will be activated resulting in expression of IFN-β. IFN-β in turn is a ligand of IFNAR (interferon α/β receptor) which can be found in the surface of the cell membrane. When IFN-β binds to IFNAR, the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway will be activated. This will result in the assembling and phosphorylation of the transcription factor ISGF3 (interferon-stimulated gene factor 3). Furthermore, ISGF3 binds to a DNA sequence called ISRE which is an enhancer element upstream.
the promoter of more than 300 different ISGs (interferon-stimulated genes).
Transcription and translation of ISGs will result in an increased production of IFN-β.
So basically, the pathway serves as a positive feedback system.

For each well, the poly (I:C) stimulation was performed as followed: 2µL of the poly (I:C) was added into 50µL of the DMEM (Dulbecco's modified eagle medium) and incubated at room temperature for 20 minutes. Thereafter, 50µL of the suspension was dripped into the well and the plate was gently shaken. Subsequently, the plate was incubated at 37°C for 18-24 hours.

**Dual Luciferase Reporter Assay**
The pISRE reporter plasmid contains a coding gene of firefly luciferase and an ISRE enhancer element which ISGF3 may bind to. If this occurs, luciferase will be expressed and by catalyzing its substrate, luciferin, light will be produced. By measuring the activity of the luciferase enzymes, i.e. the bioluminescence, inhibition of the IFN-β production could be predicted.

The Dual-Luciferase Reporter Assay System (Promega, US) was used in this step and the assay was performed on a 24-well culture plate according to the manufacturer's protocol. The luciferase activity was measured by GloMax - Multi Detection System (Promega, US).

**RESULTS**

**Reverse transcription qPCR**
RNA was extracted from the Mink and the Swan samples and thereafter, an RT-qPCR was performed to screen for the M gene which is highly conserved among influenza A viruses. RT-qPCR showed that the Mink sample had a Ct (threshold cycle) value of about 31 (*Figure 1*) while the Swan sample had a Ct value of approximately 28 (not showed). This meant that both samples were positive for influenza A virus and both were further processed.
Figure 1. Mink sample was positive for the M gene of influenza A virus after the RNA extraction. The Ct value of Mink was approximately 31.

**PCR of the NS Gene**

After having confirmed that both Mink and Swan samples were positive for influenza A virus, the RNA genome was synthesised into cDNA by RT-PCR. Thereafter, the NS gene was amplified by PCR using primers attached with restriction sites. The amplicons of these samples were labelled as Mink and Swan. The two cDNA samples were also collaterally amplified by PCR using flag tagged primers attached with restriction sites and these new flag tagged amplicons were labelled as Mink-flag and Swan-flag. Agarose gel electrophoresis was performed to ascertain that the masses of the amplicons from both PCR were correct. The gel showed bands of approximately 900bp for the Mink and the Swan samples and 700bp for the Mink-flag and the Swan-flag samples. The differences in the band sizes were due to the different primers.

**BigDye Terminator Cycle Sequencing**

The sequencing was performed three times during the proceedings of the study to detect possible modifications of the NS gene. At the first sequencing, the sequence reaction was performed for the Mink and Swan samples. The primers had the restriction sites of Xbal and Kpnl attached on the 5'-end. Thereafter the DNA was
precipitated and run on a sequencing machine. These DNA sequences were used as control templates for those samples that were sequenced later. The PBM sequence for Mink was ESKV and EPKV for Swan, respectively.

The second sequencing was performed for all four samples after the screening of recombinant colonies by colony PCR. Two different pair of primers were used in the sequencing reactions. For non-flag tagged samples (Mink & Swan) primers attached with restriction sites of KpnI and XbaI were used. For the flag tagged samples (Mink-flag & Swan-flag) the N-CMV-30 primers were used. Although Mink and Mink-flag samples originated from the same isolate the PBM sequence was different, ESKV for the Mink sample and ESEV for the Mink-flag sample. The PBM sequences, which were identified as EPKV respective ESEV, also differed between the Swan and the Swan-flag samples.

The third and last sequencing was performed after the rDNA was isolated and controlled. The PBM sequence of all samples remained unchanged, i.e. DNA sequences corresponding to ESKV, ESEV and EPKV. These PBM motifs will be compared when interpreting the result from the dual luciferase reporter assay.

**Cleavage and Ligation**

All four samples were cleaved by restriction enzymes (Xba & Kpn) and the amplicons were controlled by agarose gel electrophoresis. The result showed that the band size of the samples were unchanged for Mink, Swan and the flag tagged samples. For both vectors, a band size of about 5Kb could be seen which were the correct size of the vectors.

At the ligation step, the pcDNA3.1+ vector was ligated with the NS gene of Mink and Swan samples while the pCMV-flag vector was ligated with the NS gene of Mink-flag and Swan-flag samples. The agarose gel electrophoresis showed that no bands could be seen for all the samples which was promising because no bands should be detected if the ligation was successful. This meant that the insert had been ligated together with the vector and thus combined into a larger DNA molecule. Due to its large size and relaxed form, travelling in the agarose gel will be difficult. The
four rDNA samples were further inserted in DH5α cells by transformation and cultured.

**Screening of Recombinant Colonies**

After identification of the recombinant colonies for each sample, the rDNA was isolated and purified. The concentration of the rDNA was measured and all samples had a concentration of approximately 130 ng/μL. An *in vitro* transcription was performed by PCR with promoter primers to check if the insert was still within the rDNA. Agarose gel electrophoresis showed that the duplicates of the samples contained an insert with a band of approximately 900bp as seen in *figure 2* (only the flag tagged samples were illustrated). The rDNA bands with sizes of roughly 5Kb could also been seen.

![agarose gel electrophoresis](image)

*Figure 2. PCR was performed to control that the insert was within the rDNA. Agarose gel electrophoresis showed that the inserts were approximately 900bp and the rDNA bands were roughly 5Kb in the sample duplicates.*
**Dual Luciferase Reporter Assay**

After transfection and poly (I:C) stimulation, the activity of the firefly luciferase of the samples were measured in duplicates with empty pcDNA3.1+ vectors as negative controls (Figure 3). The bars correspond to the luciferase activity, i.e. the bioluminescence intensity; the higher value the more IFN-β will be produced.

![Figure 3](image)

*Figure 3*. The duplicate samples were analysed for the firefly luciferase activity. RFU refer to the bioluminescence emitted when luciferase catalyzed luciferin. The higher RFU value the less will the NS1 proteins suppress the IFN-β production. Empty pcDNA3.1+ vectors were used as negative controls which meant these values should be highest.

As seen in figure 3, the result showed dissimilarity among the duplicates of each sample, except for sample Swan with approximately 0.820 respectively 0.870 RFU (relative fluorescence unit).

The values of the duplicates of the negative control differed too much. Furthermore, the value for one duplicate of the negative control was the lowest of all the samples. The second duplicate of the negative control had a value below one Mink duplicate and both Swan duplicates. Therefore, the result of the dual luciferase reporter assay was unreliable since the negative controls should have had a high
The PDZ domain is an approximately 80-90 amino acids structure commonly found in cellular proteins in many living organisms. The proteins containing PDZ domain serve as scaffoldings in large complexes and are also of importance in, inter alia, signalling pathways. The PDZ domain binds to a peptide sequence termed as PBM (PDZ domain binding motif), commonly located in the carboxyl terminus of the target protein. The PBM sequence of influenza A viruses consists of a consensus sequence of four amino acids localized in the C-terminal of the NS1 protein (Javier & Rice 2011).

Zohari et al. (2010) demonstrated that allele A of the NS gene of the influenza A viruses had a greater inhibitory effect on the ISRE promoter than allele B. Therefore in the present study, we tried to determine the effects the PBM sequences of the NS1 proteins of allele A of the influenza A viruses will have on the IFN-β expression. To study how different NS1-PBM sequences will affect the IFN-β production, the PBM sequence must differ between the two isolates but the rest of the amino acids sequence should be as identical as possible. The influence of the NS1 protein was demonstrated by a reporter plasmid expressing luciferase assay under the control of ISRE. The methods used in this study are well studied and optimized for the purpose. Despite of that, some problems occurred during the proceedings.

The promoter primers, T7 and Sp6, did not work properly and the intention of using vector specific promoter primers were to see if the NS insert had successfully combined with the vector. By replacing T7 and Sp6 promoter primers with KpnI and XbaI attached primers, the NS gene could be amplified and verified. Furthermore, instead for columns from the miniprep kit, columns from PCR Clean-Up System were used to isolate the rDNA. This should not affect the outcome since the gels
were both hydrophobic.

Moreover, sequences of the NS gene of samples from same isolate showed differences of single amino acids, not only in regions upstream the PBM sequence but also in the PBM sequence itself. These genetic modifications may have been caused by the DNA polymerase. Another possible scenario was that different strains of the influenza A viruses were present in the same sample. The chance to pick two different strains from one sample was not high but could not be excluded. The amino acids sequences were not 100% identical in Mink and Swan samples. However, the minor modifications upstream the PBM sequence were not in any critical or important regions of the NS1 protein and some of the amino acids had been substituted by an amino acid with same properties. Hopefully, these differences will not result in any significant impacts on the study.

To summarize this, the PBM sequences not only differed in samples from different isolates but also in samples from the same isolate. The findings were unexpected but by having more variants of the PBM sequences the better will the study show how the PBM sequence will affect the IFN-β production.

The result obtained from the dual luciferase reporter assay showed irregular values of the duplicate samples. Only the Swan samples showed comparable values of the luciferase activity. Unfortunately, the values of the negative control (empty vectors) of the luciferase expression were low. This indicated that the measurement was incorrectly performed and thus the result can not be trusted. It was observed that the amounts of cells in the wells were less than expected after the analysis that could explained the result. The problem may arose during the measurement of the luciferase activity when we got difficulties with the measuring instrument. This because the instrument have not been used for a long period of time and was not well prepared. In addition, the preparations of the A549 cells were not either optimized since the cells need to circulate for a longer period to stabilize. The cells were thus not in condition to grow sufficiently to reproduce the transfection experiment for at least three times before the time run out.
Therefore, further experiments and investigations are needed for an accurate evaluation with the focus in solving two main tasks: One, to find out why the PBM sequences from the same isolate were different when these should be the same. Two, to repeat the transfection experiment for at least three times and also have the GloMax - Multi Detection System instrument well prepared for the dual luciferase reporter assay.

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I would like to dedicate this work to a very special person, who encouraged me and gave me the strength to accomplish the three-year plan.

QMT, thank you for believing in me. Em oi, anh doi bong woa. Di ang gèum.

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