Title: Subverting the function of PRRSV nucleocapsid protein for innovative vaccine design. NPB #09-211
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Date Submitted: June 26, 2011

Industry Summary.
Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the major virus threats facing the swine industry in the USA and worldwide. Whilst the introduction of vaccines has been moderately successful in the control of the disease, new strains are constantly emerging, especially from Asia. Live virus vaccines have been shown to be the only type of vaccine capable of establishing protective immunity against PRRSV. Design of these vaccines has been greatly helped by the establishment of the ability to genetically manipulate the virus in the laboratory setting – with the goal of altering the growth properties and/or ability of the virus to cause disease. The purpose of this project was to investigate the functioning of a virus protein, called the nucleocapsid (N) protein, which is critical for the virus life cycle. The rationale of the project was to define the biological properties of this protein in order to attenuate virus growth – and hence present candidate vaccines. Particular focus was placed on interfering with the ability of the N protein to interact with the virus genetic material (composed of a molecule called RNA). Towards this end, the research was split into three main Objectives and a contingency Objective:

Objective One was to construct a suite of recombinant PRRSV with viral RNA binding mutations in the N protein and to refine our knowledge of RNA binding. This objective involved refining RNA binding studies from Year One of the project and constructing a range of recombinant viruses whose N proteins were able to bind RNA with different efficiencies to that of N protein from wild-type virus – based on data from Year 1. The prediction being that these viruses should show a gradation of growth attenuation.

Objective Two was to characterize recombinant viruses in cell culture and compare their biology to wild type and a vaccine virus strain. The goal being to assess the degree of attenuation in these viruses compared to virus that causes disease and a similar vaccine strain.

Objective Three was to assess the stability of recombinant viruses. One of the problems associated with live-attenuated vaccines of RNA viruses (including PRRSV) is that they revert back to virulence,

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

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and this objective was to assess the stability of recombinant viruses that could be grown and recovered.

Objective Four was a contingency objective to improve the genome stability of recombinant viruses, in order to prevent reversion to wild type.
As detailed in the objectives several recombinant viruses were successfully constructed whose N proteins showed alterations in biological activity – in terms of binding to viral RNA, these focused on mutant N proteins that when expressed in isolation had less binding when compared to the normal wild type N protein. The rationale at this stage was to introduce enough changes in the N protein to prevent reversion at a latter stage. Comparison of virus biology between the recombinant viruses and the wild type virus and the vaccine virus indicated that the virus with the ability to bind viral RNA with less efficient that wild type grew less well. Those recombinant viruses which had the capability to bind RNA with approximately 15% and 25% did not effectively grow in cell culture, indicating that these changes were lethal for the virus. Extensive growing of the virus with approximately 80% RNA binding activity indicated that this property remained conserved, and no reversion to wild type occurred, thus the recombinant mutant virus was stable in cell culture. The major implications for industry are two fold. First, the biological properties of N protein can be genetically manipulated in order to attenuate virus growth – thus alterations of N protein function can be used in the design of live recombinant growth attenuated vaccines. Second, because this project has thoroughly defined the role of N protein binding to the viral genome RNA and the three-dimensional structure of the N protein is known, the possibility now arises that chemotherapeutic intervention against PRRSV can be considered and developed for emergency applications, such as the sudden emergence of virulent strains not immediately accessible to containmen through a vaccine.

Keywords:

Porcine reproductive and respiratory syndrome virus, vaccine, nucleocapsid protein, RNA binding, recombinant virus, reverse genetics.

Scientific Abstract:

The goal of this project was to investigate the biophysical properties of the porcine reproductive and respiratory syndrome virus (PRRSV) nucleocapsid (N) protein specifically focusing on its role in RNA binding and to see whether this information could be used to attenuate function in the context of a recombinant virus. Thus providing the basis for the rationale attenuation and design of live vaccines. This was a two-year project, this report focusing on Year 2. The RNA binding properties of the N protein were investigated by a combination of alanine (ala) scanning mutagenesis (reported in Year 1) and site specific mutation (Year 2) and these were mapped onto a three dimensional structure of the N protein, derived from information obtained by X-ray crystallography, circular dichroism, and molecular modelling (the latter two from this study and reported in Year 1). Together, the data confirmed a previously characterised RNA-binding domain and found several new ones as well as identifying individual amino acids that are critical for this process. The results indicated that disulfide bridge formation played a key role in RNA binding, offering an explanation why infectious virus could not be rescued if cysteine (cys) residues are mutated. Overall, the data demonstrated that multiple sites promoted RNA binding. This biophysical information was used to construct various recombinant viruses that contained defined mutations in the N gene that when expressed would lead to a reduction in RNA binding capability. The rationale being that this would result in growth deficiencies. In order to do this the N gene was sub-cloned such that a three way overlapping PCR was used to introduce selected mutations into the N gene. This cassette was then used to replace the wild type N gene sequence in the context of the full-length infectious clone of PRRSV. Thus a suite of infectious clones containing N gene mutations were constructed such that the N protein would exhibit 0% (as a

control), and then less efficient RNA-binding activity compared to wild type N protein. The backbone recombinant virus utilized was from the FL12 recombinant infectious clone of the highly virulent American PRRSV isolate (NVSL 97-7895). The ability of these viruses to be rescued and their growth kinetics (where appropriate) was determined on MARC-145 cells, which are permissive for PRRSV. Both virus titre and western blot was used at defined time points to compare the growth of the recombinant viruses with the virulent FL12 parental strain and the Prime Pac vaccine strain. Together this data indicated that the recombinant viruses expressing N proteins with various abilities to bind N protein had different growth phenotypes. As would be predicted the virus with the recombinant N protein that could not bind N protein was not rescued, as were the viruses where the binding activities were 15% and 25% of wild type. The virus with 80% RNA binding activity grew less well than the parental and vaccine strains, but could be recovered. Serial passage and sequencing of progeny virus indicated that the RNA-binding mutations in the N protein were stable. Together the data indicates that there may be a threshold level of activity of N protein and that alteration of the function of N protein can be used to construct growth deficient viruses.

INTRODUCTION:

Porcine reproductive and respiratory syndrome virus (PRRSV) is recognized as one of the major virus threats facing the swine industry in the USA and worldwide. Whilst the introduction of vaccines has been moderately successful in the control of the disease, new strains are constantly emerging. Live virus vaccines have been shown to be the only type of vaccine capable of establishing protective immunity against PRRSV.

Indeed, currently the most efficient way to produce live virus vaccines is to create stable recombinant live virus vaccines with altered growth phenotypes, yet are still capable of generating a protective immune response. Identifying and characterizing unique and important stages in the virus life cycle can achieve this, by attenuating the efficiency of virus protein function through targeted mutagenesis coupled to reverse genetic strategies. The objective of this research project to industry was to exploit knowledge of the virus nucleocapsid (N) protein to subvert virus biology and to demonstrate that altering the functionality of N protein could be used to attenuate virus replication. This was a two year project and this report is for the second year. Year 2 initially focused on refining the mapping of RNA binding using mutation and then focused on constructing and characterising the growth of recombinant viruses whose N proteins had different RNA binding capabilities. This project used the N gene/protein of the highly virulent American PRRSV isolated NVSL 97-7895, and recombinant viruses were derived from this parental strain (called FL12). The project was a unique collaboration between the University of Leeds and the Nebraska Center for Virology where our expertise in characterizing viral nucleocapsid proteins and investigating infectious recombinant viruses both *in vitro* and *in vivo* was highly complementary.

The proposed research was designed to fit within the research priorities stated in the call for proposal specifically addressing Swine Health-PRRS research objective PRRSV Immunology, subsection ‘Innovative vaccine development’. One of our major research findings was that the only effective way to establish protective immunity against PRRSV is to use a live virus vaccine. The most efficient way to design new vaccines is to exploit reverse genetic strategies. Here selected genotypic and phenotypic were introduced into a full-length infectious clone of the virus and used to generate stable infectious virus which exhibited growth attenuation. The research demonstrated that altering the functioning of N protein can be used to attenuate virus biology and hence deliver potential growth attenuated live recombinant viruses. Vaccination is an efficient and cost effective way of removing, controlling and counteracting the devastating impact of PRRSV. The development of growth attenuated live virus candidate vaccines is perhaps the one area where basic research can be directly delivered into an available product for the pork industry. The research in this grant has advanced the basic science of PRRSV focusing on the function of the N protein but has also served as a model and paradigm as to how such research can be applied to the development of growth attenuated viruses,

for possible use as vaccine candidates.

OBJECTIVES:

Objective One: Constructing a suite of recombinant PRRSV with viral RNA binding attenuating mutations in the N protein.

Objective Two: To characterize recombinant viruses in cell culture and compare their biology to wild type (FL12) and vaccine virus (Prime Pac) strains.

Objective Three: Assessing the stability of recombinant viruses.

Objective Four: Was a contingency Objective and depended on the genome stability of recombinant viruses constructed in Objective One.

MATERIALS AND METHODS:

Cells and viruses. MARC-145 cell was cultured and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heated-inactivated fetal bovine serum (FBS), 0.25 μg/ml fungizone, 100 U/ml penicillin, 10 μg/ml streptomycin sulfate, and 5 μg/ml gentamicin and then held at 37°C in a humidified 5% CO_2 incubator. Highly virulent North American PRRSV strain FL12 recombinant and Prime Pac was propagated in MARC-145 cells (Zuckermann et al. 2007).

Construction and rescue of recombinant viruses. All plasmids were propagated in E. coli DH5α and were based on FL12 (an infectious clone of strain NVSL 97-7895). The plasmids were linearized by restriction with AclI and used for in vitro transcription by T7 RNA polymerase to synthesize capped RNAs using the mMESSAGE mMACHINE Ultra T7 kit (Ambion) with cap analog. The reaction mixture was digested with DNaseI to remove template DNA and subsequently was extracted with phenol–chloroform. RNA was precipitated with an equal volume of isopropanol and recovered by centrifugation. The RNA pellet was re-suspended in water. RNA was electroporated into cells.

Western blot analysis. Protein lystates were prepared from mock-treated and cells infected with different recombinant viruses and defined points post-infection. These were analysed by immuno-blot using appropriate virus specific antibodies and antibodies to control host cell proteins such as GAPDH.

RT-PCR analysis. Total cellular RNA was extracted from MARC-145 cells by using TRIzol (Invitrogen), and treated with DNase I (Promega) according to the manufacturer's instructions. Gene specific primers to PRRSV was used to detect viral RNA.

RNA-binding analysis. Surface Plasmon resonance was used to measure the formation of N protein/RNA complexes in real-time, at physiological temperatures and simultaneously provide equilibrium and kinetic information of their binding (including association rate, disassociation rate and binding affinity) (Chen et al. 2005, Spencer and Hiscox 2006). This was complemented using gel based RNA-binding assays.

Statistical analysis. Student's *t*-test was used to determine statistical significance and *P*-values of <0.05 were considered statistically significant.

RESULTS:

Objective One: Constructing a suite of recombinant PRRSV with viral RNA binding attenuating mutations in the N protein. As detailed the focus of this Objective was to construct recombinant PRRSV with mutations in N protein that either abrogated or modulated RNA binding activity. As a first step we completed the RNA-binding study reported on for Year 1 and tested the RNA binding properties of a N protein which contained a serine for cys mutation at position 23. The rationale for doing this was to generate a completely negative control – i.e. a N protein that could not bind RNA. This was based on our data that addition of DTT (a reducing agent) in an RNA binding assay reduced RNA binding (Figure 1A). Addition of DTT disrupts disulfide bridge formation that is mediated by cys residues. Based on our structural prediction of the N protein (Figure 1B) we predicted this was the cys at position 23. Indeed substitution of this position with serine abrogated RNA-binding (Figure 1C).

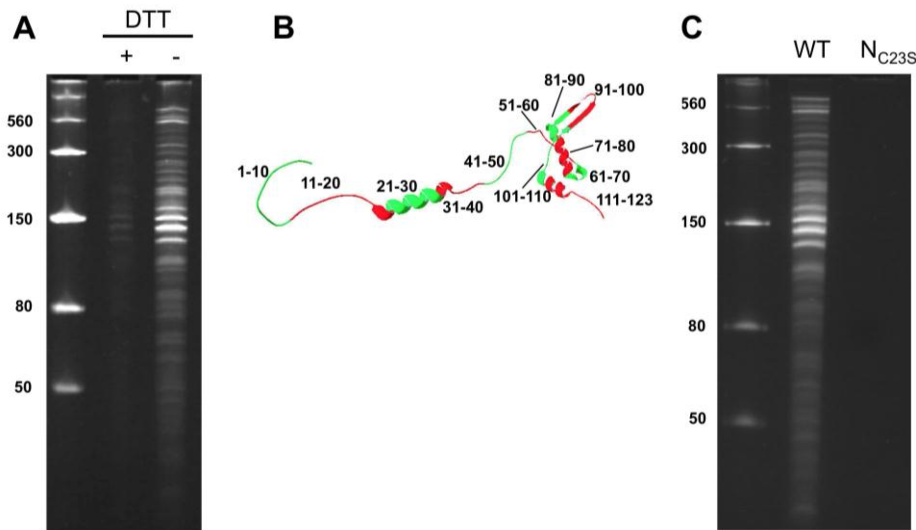

Figure 1. *(A). RNA binding is influenced by disulfide bond formation. Shown are denaturing acrylamide gels onto which RNA was loaded which was extracted from equal amounts of protein. RNA extracted from wild type N protein purified under reducing (+DTT) or non-reducing (-DTT) conditions. (B). Predicted structure of PRRSV N protein which harbors one structured domain that is located in the C-terminal half. The N-terminal half of the protein is thought to be mainly unstructured besides a predicted α–helix. Shading shows the positioning of the alanine block substitutions used in this study on the predicted structure. Cys23 occurs in the green shaded area (21-20). (C). RNA extracted from wild type N protein and the cys to serine mutant N_{C23S} that were purified under non-reducing conditions. Sizes of marker bands (bp) are indicated on the left of the panels.*

Thus taken together with work reported on in Year 1 we had generated a suite of recombinant N proteins with differential RNA-binding activity and fully mapped the RNA-binding motifs on this protein, thus adding to available knowledge and the previously characterised binding site between residues 37 and 57. Thus we found that at least five different sites in N protein mediated RNA-binding to some degree with cys23 also playing a crucial role (Figure 2). Thus suggesting that multiple sites contributed to RNA binding in N protein.

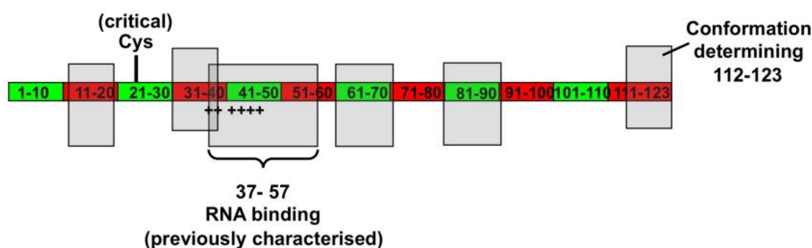

Figure 2. *Linear map of the N protein showing regions involved in RNA-binding (gray shade) discovered in this study (with the exception of amino acids 37-37). Crucial to RNA-binding was also the cys at position 23 and the C-terminus of the protein for oligomerisation. The data indicated that multiple sites of RNA-binding may act cooperatively together. The altering red and green shading indicates blocks of ten ala substitutions that were introduced into N protein for RNA activity studies.*

Therefore, the next stage involved introducing ala substitution mutations into the N gene of the full length virus, such that when the protein was expressed in the context of infection, would lead to attenuation of RNA-binding. The backbone recombinant virus utilized was from the FL12 recombinant infectious clone of the highly virulent American PRRSV isolate (NVSL 97-7895), this was also the virus from which the N protein for biophysical analysis and RNA-binding was derived. Our initial approach was to try to introduce ala substitutions directly into the FL12 vector. However, this proved unsuccessful, probably due to the size of the construct. Therefore, rather than try to introduce mutations directly into the recombinant infectious clone, we adopted a cassette approach, so that N gene mutants could be directly restricted into the backbone vector.

The basic cassette consisted of a fragment digested from FL12 (encompassing the entire N gene and upstream and downstream sequence) using the unique restriction enzymes AclI and MfeI (Figure 3A), this was then sub-cloned into vector pCR2.1 for stability, and confirmed by sequencing in both orientations. This vector was then used as a template for over-lapping PCR to introduce the alanine substitution mutations (Figure 3B). The mutations selected were $N_{81\text{-}90A}$, $N_{71\text{-}80A}$, $N_{11\text{-}20A}$, $N_{51\text{-}60A}$ and N_{C23S}. Based on the biophysical studies these were predicted to give approximately 80%, 50%, 25%, 15% and 0% RNA-binding activity. The mutated cassettes were then substituted back into FL12 creating recombinant virus and sequenced. These recombinant plasmid were termed pFL12$N_{81\text{-}90A}$, pFL12$N_{71\text{-}80A}$, pFL12$N_{11\text{-}20A}$, pFL12$N_{51\text{-}60A}$ and pFL12N_{C23S}. *In vitro* transcription of the genomic RNA template for electroporation was confirmed for each recombinant plasmid after RNA clean up (Figure 3C). This successfully completed Objective 1.

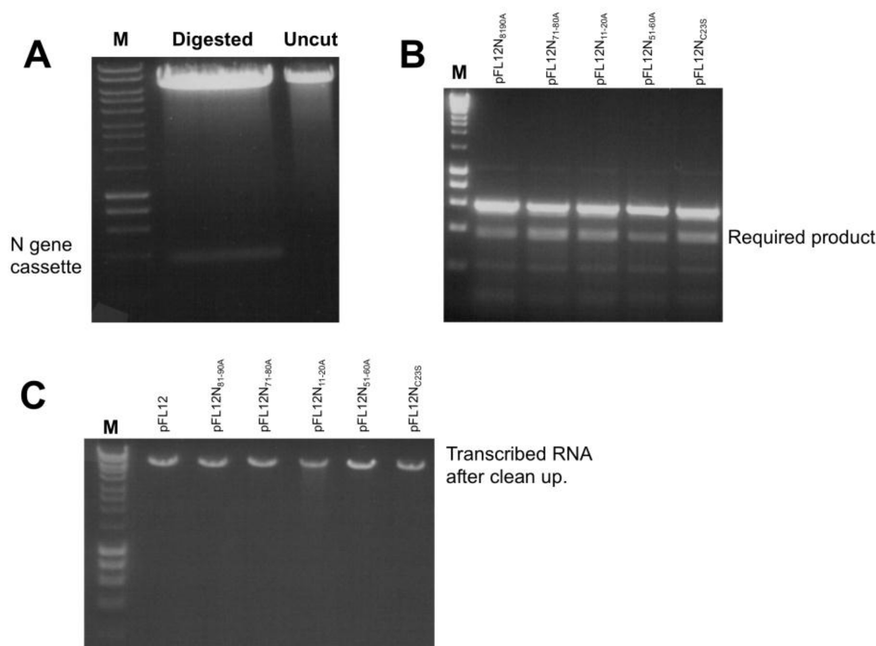

Figure 3. Major check stages in the cloning of recombinant PRRSV that encode N proteins with deficient RNA binding capability. (A). Agarose gel electrophoresis analysis of the FL12 plasmid digested with the enzymes AclI and MfeI to generate an N gene cassette for subsequent sub-cloning. (B). Agarose gel electrophoresis analysis of the products in the two-step PCR for the generation of alanine substitutions in the N protein. For example, note that pFL12$N_{81\text{-}90A}$ contains coding mutations such that amino acids between positions 80 and 90 in the context of the N protein are substituted for

ala. (C). Virus is rescued from in vitro transcribed RNA electroporated into cells. This agarose electrophoresis analysis of in vitro transcribed RNA products (after clean up by DNase treatment and purification) from the plasmids with different N gene mutations based on the FL12 backbone.

Objective Two: To characterize recombinant viruses in cell culture and compare their biology to wild type (FL12) and vaccine virus (Prime Pac) strains.

The focus of this Objective was to compare the growth properties of the recombinant viruses, $pFL12N_{81\text{-}90A}$, $pFL12N_{71\text{-}80A}$, $pFL12N_{11\text{-}20A}$, $pFL12N_{51\text{-}60A}$ and $pFL12N_{C23S}$ to the wild type parental virus and a vaccine strain. This first involved rescue of the virus in cell culture and then comparison of growth properties using one-step growth curves, western blot analysis and immunofluorescence as independent checks on this. To potentially rescue the recombinant viruses the purified *in vitro* transcribed RNA was electroporated into MARC-145 cells, using protocols that had previously been used to rescue the FL12 recombinant virus (Truong et al. 2004). At 48 hours post-electroporation supernatant was collected and clarified by centrifugation, and this was used to infect naïve MARC-145 cells in 35-mm dishes. Growth kinetics were examined by removing media at selected times points post-infection with the recombinant viruses and virus titres were determined by plaque assay on MARC-145 cells and expressed as plaque forming unit per millilitre (PFU/ml). An example of virus plaques from the parental FL12 strain and the various potential recombinant viruses derived from the rescue of RNA *in vitro* transcribed from $pFL12N_{81\text{-}90A}$, $pFL12N_{71\text{-}80A}$, $pFL12N_{11\text{-}20A}$, $pFL12N_{51\text{-}60A}$ and $pFL12N_{C23S}$ is shown in Figure 4A.

The data indicated that growth/rescue efficient was related to the RNA-binding capability of the expressed N protein. RNA transcribed from $pFL12N_{81\text{-}90A}$ and $pFL12N_{71\text{-}80A}$ was less efficiently rescued than RNA transcribed from the parental pFL12. RNA transcribed from $pFL12N_{11\text{-}20A}$ and $pFL12N_{51\text{-}60A}$ generated very few plaques and RNA transcribed from $pFL12N_{C23S}$ appeared not to be rescued, at least determined by the absence of plaques. For RNA transcribed from $pFL12N_{C23S}$, the positive control, this would be predicted, as recombinant PRRSV containing substitutions for cys residues, including cys23, has previously been described to be lethal (Lee et al. 2005), and we can now conclude this is due to deficiencies in binding RNA.

To confirm that the cells had been electroporated with the *in vitro* transcribed RNA we used RT-PCR to detect the genomic RNA and GAPDH as a control to show successful RNA extraction and equal loading. The data indicated that for all constructs input RNA could be detected (Figure 4B). However, 48 h hours post-electroporation genomic RNA could still be detected for RNA transcribed from $pFL12N_{81\text{-}90A}$, $pFL12N_{71\text{-}80A}$ and $pFL12N_{11\text{-}20A}$, with some signal visible for $pFL12N_{51\text{-}60A}$. No genomic RNA was detected for $pFL12N_{C23S}$ (Figure 4B). Again the GAPDH RT-PCR control demonstrated total RNA purification was successful and there was equally loading across all samples (Figure 4B). As an independent investigation of this total cellular lysates were prepared at 48 hr post-electroporation and western blot using an antibody to N protein was used to investigate the amount of N protein after rescue. The data indicated that N protein was detected for rescue of RNA from $pFL12N_{80\text{-}90A}$ but less for $pFL12N_{71\text{-}80A}$, $pFL12N_{11\text{-}20A}$ and $pFL12N_{51\text{-}60A}$ with no signal for $pFL12N_{C23S}$ (Figure 4C). Again GAPDH was used to demonstrate sample preparation and equal protein loading controls (Figure 4D). The elctrophoretic mobility of the modified N protein (with ten alanine substitutions) is slightly slower than the wild-type N protein, and this was similar to that reported on in Year 1, and can be attributed to the slightly different molecular weight and charge.

Taken together this data indicated that abrogation of RNA-binding is lethal for virus replication ($pFL12N_{C23S}$) and confirms available literature (Lee et al. 2005). Reducing the efficiency of RNA binding also results in reduction of viral growth as determined by several different assays. RNA

transcribed from $pFL12N_{81\text{-}90A}$ and $pFL12N_{71\text{-}80A}$ could be rescued with RNA transcribed from $pFL12N_{81\text{-}90A}$ showing increased efficiency. This was investigated independently of plaque assay, RT-PCR or western blot using indirect immuno fluorescence confocal microscopy with sera to detect PRRSV proteins. Coverslips were prepared 48 hr post-electroporation and fixed using formaldehyde and made permeable. The data indicated that viral proteins could be detected in cells electroporated with RNA transcribed from $pFL12N_{81\text{-}90A}$, $pFL12N_{71\text{-}80A}$ and $pFL12N_{11\text{-}20A}$ (Figure 4D) but was below the limits of detection in the linear range for RNA transcribed from $pFL12N_{11\text{-}20A}$ (e.g. Figure 4D), $pFL12N_{51\text{-}60A}$ and $pFL12N_{C23S}$. Comparison of growth curves between the different recombinant viruses and the parental FL12 and Prime Pac vaccine strain of PRRSV also indicated that virus derived from $pFL12N_{81\text{-}90A}$ had approximately a two log difference with the virus derived from FL12 parental recombinant virus (Figure 4E). Virus from RNA transcribed from $pFL12N_{11\text{-}20A}$, $pFL12N_{51\text{-}60A}$ and $pFL12N_{C23S}$ were below the limits of resolution. Previous data indicated that FL12 and a recombinant derived Prime Pac vaccine had equivalent growth kinetics (Kwon et al. 2006).

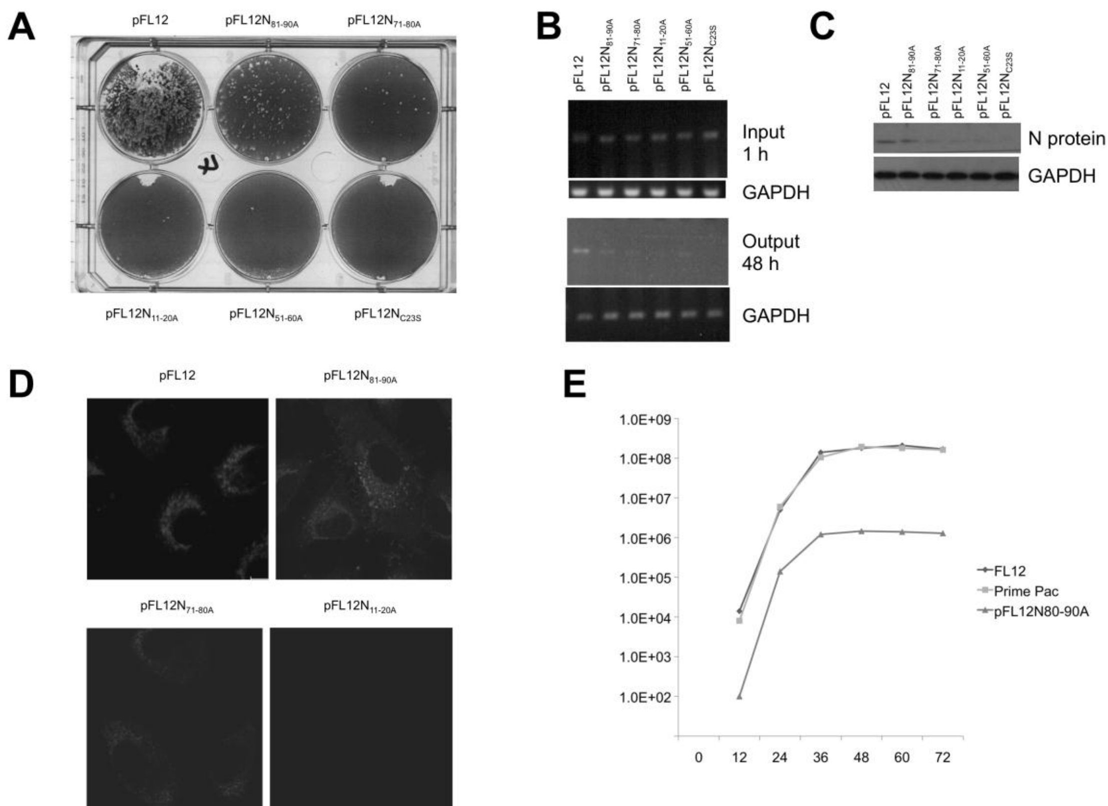

Figure 4. (A). Example of a plaque assay comparison of recombinant viruses expressing N proteins with different RNA-binding activity compared to the parental virus (FL12). (B). RT-PCR analysis of input electroporated RNA to show that the RNA has been introduced into MARC-145 cells for rescue and subsequent RT-PCR analysis 48 h later to determine whether genomic RNA is still present – as a replication product. (C). Western blot analysis of the N protein in MARC-145 cells infected with the various recombinant viruses. (D). Immunofluorescence analysis showing examples of cells infected with the different recombinant viruses as indicated. Viruses with low RNA binding activity of N protein and that could not be detected with RT-PCR and western blot were below the limits of detection with confocal. (E). Example of a growth curve comparing the growth curves of RNA transcribed from recombinant FL12, Prime Pac and $pFL12N_{81\text{-}90}$.

Objective Three: Assessing the stability of recombinant viruses.

One of the difficulties with PRRSV live vaccines and PRRSV in general is the high mutation rate. This is a general property of RNA viruses and is responsible for wide sequence divergence between different virus strains. The practical implication from a live vaccine perspective is that these viruses

can revert to virulence. The error rate of an RNA-dependent RNA-polymerase is approximately 10^5 and therefore recombinant viruses with nucleotide substitutions that alter coding (and hence virus protein function) have a selection pressure to revert to functionality. This was previously observed in a recombinant PRRSV whose N protein contained mutations in the nuclear localisation signal in N protein that led to loss of function in nuclear localisation (an essential property of the N protein) (Lee et al. 2006).

With passage in cell culture and also experimentally *in vivo* functionality (i.e. nuclear localisation) was restored through nucleotide substitutions recreating the nuclear localisation signal – through selection pressure (Lee et al. 2006, Pei et al. 2008). With this important and defining study in mind we took the design philosophy that loss in efficiency of function rather than abrogation would lower or eliminate selection pressure. In addition, reducing the RNA-binding efficiency of N protein was achieved through the substitution of blocks of ten amino acids with ala residues. For example, with pFL12N_{81-90A}, which was the only recombinant virus in this study to be efficiently rescued, this resulted in the replacement of eight residues for ala, as there were already two ala residues between positions 81 to 90 (Figure 5). Examples of the nucleotide changes introduced to create these substitutions are also shown (Figure 5). The recombinant virus derived from RNA transcribed from pFL12N_{81-90A} was serially passaged fifteen times in MARC-145 cells by collecting supernatent at 72 hrs post-infection for each passage. This was also used to infect the next passage that was infected at the same MOI and so on. RT-PCR and western blot analysis indicated no significant change in the levels of genomic RNA or protein with each passage and the peak titre at 72 hrs post-infection was in the range of 1.0E+06 with no reversion to wild type (approximately in the range of 1.0E+08). This indicated that the mutation in N protein was stable from a growth perspective and this was confirmed by sequencing the N gene at each passage.

FL12 N protein sequence

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MPNNNGKQQK KKRGNGQPVN QLCQMLGKII AQQNQSRGKG PGKKIKNKNP
EKPHFPLATE DDVRHHFTPS ERQLCLSSIQ TAFNQGAGTC TLSDSGRISY
TVEFSLPTHH TVRLIRVTAP SSA
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pFL12N_{81-90A} N protein sequence

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MPNNNGKQQK KKRGNGQPVN QLCQMLGKII AQQNQSRGKG PGKKIKNKNP
EKPHFPLATE DDVRHHFTPS ERQLCLSSIQ AAAAAAAAAA TLSDSGRISY
TVEFSLPTHH TVRLIRVTAP SSA
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ATG CCA AAT AAC AAC GGC AAG CAG CAA AAG
AAA AAG AGG GGG AAT GGC CAG CCA GTC AAT
CAG CTG TGC CAG ATG CTG GGT AAG ATC ATC
GCC CAG CAA AAC CAG TCC AGA GGC AAG GGA
CCG GGG AAG AAA ATT AAG AAT AAA AAC CCG
GAG AAG CCC CAT TTT CCT CTA GCG ACT GAA
GAT GAC GTC AGG CAT CAC TTC ACC CCT AGT
GAG CGG CAA TTG TGT CTG TCG TCG ATC CAG

ACT GCC TTT AAC CAG GGC GCT GGA ACC TGT   <- Codon sequence for FL12
GCC GCC GCC GCC GCC GCT GCT GCC GCT GCC   <- Codon sequence for pFL12N81-90A

ACC CTA TCA GAT TCA GGT AGG ATA AGT TAC
ACT GTG GAG TTT AGT TTG CCG ACG CAT CAT
ACT GTG CGC CTG ATC CGC GTC ACA GCG CCA
TCA TCA GCG TAA(stop)
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Figure 5. Amino acid and corresponding nucleotide substituions used to create the N protein RNA binding mutant expressed from pFL12N_{81-90A}.

Objective Four: Was a contingency Objective and depended on the genome stability of recombinant viruses constructed in Objective One.

As pFL12N_{81-90A} was stable upon passage, there was no need for this contingency Objective.

Discussion:

Both Year 1 (previously reported) and Year 2 (this report) were focused on testing the hypothesis that altering the RNA-binding efficiency of N protein could be used as a way of generating growth deficient PRRSV to provide proof of concept that altering the function(s) of PRRSV N protein can be used to generate growth attenuated live recombinant viruses. Year 2 of the study completed the RNA binding analysis and then focused on the generation and characterisation of recombinant viruses with deficiencies in the ability of N protein to bind RNA. The data indicated that unlike the gradational ability of N protein mutants to bind RNA with different efficiencies, only one mutant had appreciable growth characteristics – virus derived from $pFL12N_{81\text{-}90A}$, as predicted these were less than wild type virus – thus demonstrating growth attenuation through deficiencies in RNA-binding activity. Previous studies reported in Year 1 through the use of EGFP-tagged wild type N protein and mutants demonstrated that (with the exception of the N-terminal 10 amino acids) the mutant N proteins were stable in cell culture and did not aggregate, thus we have confidence that virus-attenuation was through deficiency in RNA binding activity rather than protein stability. Thus the central hypothesis has been answered – that the functionality of the PRRSV N protein can be altered to generate stable recombinant infectious virus that is growth attenuated compared to wild type virus.

This work has been presented at several national and international meetings and invited seminars in its different stages and has lead to further grant funding to study PRRSV N protein (a personal Leverhulme Trust Research Fellowship to JAH) and also a collaboration between the Hiscox laboratory and the laboratory of Prof. Hongying Chen at Northwest A&F University in China to develop codon de-optimised PRRSV live recombinant vaccines, based on the N protein work. The results of Years 1 and 2 have been incorporated into two manuscripts that have been submitted for publication (enclosed with this report). The first paper describes the RNA-binding studies on recombinant N protein and mutants (NPB is acknowledged as the sole funding agency). The second paper describes the use of the EGFP-tagged proteins generated in Year 1 (to assess N protein stability) in generating a cellular proteome interactome map (as part of and funded by the Leverhulme Fellowship, again NPB is acknowledged).

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