

2009

# Characterization of Infectivity and Pathogenesis of Partially Reconstructed 1918 and Highly Pathogenic Avian Influenza Viruses In The BALB/C Mouse Model

John Allen Pyles  
*Wright State University*

Follow this and additional works at: [https://corescholar.libraries.wright.edu/etd\\_all](https://corescholar.libraries.wright.edu/etd_all)



Part of the [Pharmacology, Toxicology and Environmental Health Commons](#)

---

## Repository Citation

Pyles, John Allen, "Characterization of Infectivity and Pathogenesis of Partially Reconstructed 1918 and Highly Pathogenic Avian Influenza Viruses In The BALB/C Mouse Model" (2009). *Browse all Theses and Dissertations*. 924.  
[https://corescholar.libraries.wright.edu/etd\\_all/924](https://corescholar.libraries.wright.edu/etd_all/924)

This Thesis is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact [corescholar@www.libraries.wright.edu](mailto:corescholar@www.libraries.wright.edu), [library-corescholar@wright.edu](mailto:library-corescholar@wright.edu).

CHARACTERIZATION OF INFECTIVITY AND PATHOGENESIS OF PARTIALLY  
RECONSTRUCTED 1918 AND HIGHLY PATHOGENIC AVIAN INFLUENZA  
VIRUSES IN THE BALB/c MOUSE MODEL

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

By

JOHN ALLEN PYLES

B.S., Ohio State University, 2002

2009  
Wright State University

WRIGHT STATE UNIVERSITY  
SCHOOL OF GRADUATE STUDIES

March 11, 2009

I HEREBY RECOMMEND THAT THE THESIS PREPARED  
UNDER MY SUPERVISION BY John Allen Pyles ENTITLED  
Characterization of Infectivity and Pathogenesis of Partially  
Reconstructed 1918 and Highly Pathogenic Avian Influenza Viruses  
in the BALB/c Mouse Model BE ACCEPTED IN PARTIAL  
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE  
OF Master of Science

---

Carol Sabourin, Ph.D.  
Thesis Director

---

Mariana Morris, Ph.D.  
Department Chair

Committee on  
Final Examination

---

Carol Sabourin, Ph.D.

---

David Cool, Ph.D.

---

Courtney Sulentic, Ph.D.

---

Joseph F. Thomas, Jr., Ph.D.  
Dean, School of Graduate Studies

## ABSTRACT

Pyles, John Allen. M.S., Department of Pharmacology and Toxicology, Wright State University, 2009.

Characterization of Infectivity and Pathogenesis of Partially Reconstructed 1918 and Highly Pathogenic Avian Influenza Viruses in the BALB/c Mouse Model.

Influenza viruses are consistently responsible for an average of 20,000 deaths and 114,000 hospitalizations per year. To a great extent, these viruses always stay one step ahead of the available vaccines and people's immunity year after year because they have the ability to either mutate part of their genetic material, or to be transmitted from one species to another. That same genetic variability explains why highly pathogenic influenza viruses emerge that cause great mortality over several countries resulting in pandemics. Highly pathogenic strains of influenza A virus have emerged occasionally in recent history, producing pandemics such as the one in 1918. The Spanish influenza pandemic of 1918–1919 was uniquely severe, causing an estimated 50 million deaths worldwide. Also unique was the age distribution of its victims: the death rate for young, previously healthy adults, who rarely suffer fatal complications from influenza, was exceptionally high. More recently we have seen the emergence of influenza cases and fatalities involving the H5N1 avian influenza strains. Until an outbreak in Hong Kong claimed six human lives in 1997 (A/Hong Kong/156/97 [H5N1]), avian influenza viruses were thought to be incapable of infecting humans directly. However, the initial H5N1 outbreak has revealed that avian influenza viruses could infect humans without prior adaptation and even cause significant morbidity and mortality in the human population. It

has been shown that the 1918 viral hemagglutinin sequence is more closely related to avian strains although it is a human HA. This indicates that the 1918 pandemic strain may have also jumped from avian to human with no prior adaptation or reassortment with a human virus.

For these reasons, scientists have been interested in finding out what makes the 1918 virus different from all others, why avian influenza can be so pathogenic, and how to prevent and better treat infections with this virus.

For this study the 1918 pandemic influenza was partially reconstructed by placing the 1918 influenza viral hemagglutinin and neuraminidase genes or the 1918 influenza viral hemagglutinin, neuraminidase and non-structural protein genes in the background of the low-pathogenic A/Texas/36/91 virus. The infectivity and pathogenesis of these two partially reconstructed 1918 influenza viruses were compared to each other as well as to the highly-pathogenic avian influenza A/Vietnam/1203/04 H5N1 virus in the BALB/c mouse model. The A/Vietnam/1203/04 influenza virus acted as a “positive” control for high infectivity and pathogenesis.

## Table of Contents

List of Figures.....	vi
List of Tables .....	vii
Abbreviations.....	viii
Introduction.....	1
Background.....	3
Hemagglutinin (HA).....	5
Neuraminidase (NA).....	10
Non-Structural Protein (NS).....	12
Reverse Genetics.....	13
Mice Influenza Model.....	16
Study Objectives .....	17
Materials and Methods.....	18
Results.....	25
Statistical analysis of TCID <sub>50</sub> Data .....	33
Discussion.....	36
Summary .....	40
References.....	41

## List of Figures

Figure	Page
1. Schematic of the plasmid-based rescue system . . . . .	19
2. pCAGGS-PB1/PB2/PA/NP/GFP control transfection . . . . .	25
3. Lethality determination of 1918 HA/NA:TX91 . . . . .	27
4. Lethality determination of 1918 HA/NA/NS:TX91 . . . . .	27
5. Time course of mortality following challenge . . . . .	28
6. Change in body weight following challenge . . . . .	29
7. Mean viral lung titers . . . . .	29

## List of Tables

Table	Page
1. Stock influenza viral titers and challenge doses . . . . .	24
2. Lung titers at 24, 72, and 120 hours post-challenge . . . . .	32
3. Summary of ANOVA results and Tukey's Pairwise . . . . .	34
4. Reduced data set of ANOVA results and Tukey's Pairwise . . . . .	35



## Abbreviations

1918 HA/NA:TX91 – partially-reconstructed virus containing the HA and NA genes of the 1918 pandemic strain with the remaining six genes from A/Texas/36/91

1918 HA/NA/NS:TX91 - partially-reconstructed virus containing the HA, NA and NS genes of the 1918 pandemic strain with the remaining five genes from A/Texas/36/91

293T – Human Embryonic Kidney 293 cells

ANOVA - analysis of variance

AV18 – A/South Carolina/1/18 strain with amino acid mutations at site 190 and 225

A/Vietnam – A/Vietnam/1203/2004

Brevig/18 – A/Brevig Mission/1/18

BSL-3+ - biosafety level 3 enhanced

CAT - chloramphenicol acetyltransferase

cDNA – complementary deoxyribonucleic acid

dsRNA – double-stranded ribonucleic acid

eIF2 $\alpha$  - eukaryotic translation initiation factor 2

GFP – green fluorescent protein IRF-3 – interferon regulatory factor 3

HA – hemagglutinin

HMG – hydroxymethylglutaryl-coenzyme A reductase

IFN – interferon

IFN- $\alpha$  – interferon alpha

IFN- $\gamma$  – interferon gamma

IRF-3 – interferon regulatory factor 3

K173 - A/Kawasaki/173/2001

LD<sub>50</sub> – fifty percent lethal dose

LOD - limit of detection

M – matrix

M88 - A/Memphis/8/88 (M88)

MDCK - Madin-Darby canine kidney

MEM – minimum essential medium

NA – neuraminidase

NEP – one of two proteins encoded by the NS gene

NF- $\kappa$ B – nuclear factor kappa-light-chain-enhancer of activated B cells

NP – nucleoprotein

NS – non-structural

NS1 – one of two proteins encoded by the NS gene

NY18 – A/New York/1/18

ORF - open reading frame

PA – polymerase protein

PB1 - viral transcriptase

PB2 - viral endonuclease

PBS – phosphate buffer saline

p.c. – post challenge

PFU – plaque forming units

p.i. – post-infection

PKR – protein kinase R

polI - RNA polymerase I

SC18 – A/South Carolina/1/18

SJPL - St. Jude porcine lung

TCID<sub>50</sub> - fifty percent tissue culture infectious dose

TGF- $\beta$  – transforming growth factor beta

TNF- $\alpha$  – tumor necrosis factor alpha

TX91 – A/Texas/36/91

vDNA – viral deoxyribonucleic acid

WSN - A/WSN/33

## Introduction

Influenza viruses are consistently responsible for an average of 20,000 deaths and 114,000 hospitalizations per year. To a great extent, these viruses always stay one step ahead of the available vaccines and people's immunity year after year because they have the ability to either mutate part of their genetic material, or to be transmitted from one species to another. That same genetic variability explains why highly pathogenic influenza viruses emerge that cause great mortality over several countries resulting in pandemics. Highly pathogenic strains of influenza A virus have emerged occasionally in recent history, producing pandemics such as the one in 1918. The Spanish influenza pandemic of 1918–1919 was uniquely severe, causing an estimated 50 million deaths worldwide (1, 2). Also unique was the age distribution of its victims: the death rate for young, previously healthy adults, who rarely suffer fatal complications from influenza, was exceptionally high (1, 3). More recently we have seen the emergence of influenza cases and fatalities involving the H5N1 avian influenza strains.

Until an outbreak in Hong Kong claimed six human lives in 1997 (A/Hong Kong/156/97 [H5N1]), avian influenza viruses were thought to be incapable of infecting humans directly. However, the initial H5N1 outbreak has revealed that avian influenza viruses could infect humans without prior adaptation and even cause significant morbidity and mortality in the human population. It has been shown that the 1918 viral hemagglutinin (HA) sequence is more closely related to avian strains although it is a human HA (1). This indicates that the 1918 pandemic strain may have also jumped from avian to human with no prior adaptation or reassortment with a human virus (1).

For these reasons, scientists have been interested in finding out what makes the 1918 virus different from all others, why avian influenza can be so pathogenic, and how to prevent and better treat infections with this virus.

The purpose of this study is to compare the infectivity and lethality of two recombinant 1918 pandemic influenza viruses; one containing the HA and neuraminidase (NA) genes of the 1918 influenza virus and another containing the HA, NA and non-structural proteins (NS) genes of the 1918 influenza virus. These genes will be placed in the background of the low pathogenic Texas/91 influenza strain. The lethality and infectivity of these two recombinant 1918 viruses will be compared to each other as well as to the parent Texas/91 influenza virus and the A/Vietnam/1203/04 H5N1 avian influenza viruses in the mouse model.

## Background

Influenza is a multi-partite virus containing 7-8 negative-stranded RNA genomic segments. These RNA segments code for ten proteins: hemagglutinin (HA), neuraminidase (NA), non-structural proteins (NS1 and NEP), polymerase proteins (PB1, PB2 and PA), matrix proteins (M and M2) and nucleoprotein (NP). Since the influenza genome is in the negative sense it has to provide its own RNA-dependant RNA polymerase in order to make RNA capable of being read by eukaryotic ribosomes. This polymerase complex lacks proofreading capability, such that one in five virus particles produced is likely to contain a change at one of its approximately 13,500 nucleotides (18). If such a change provides the virus with a competitive advantage, that strain quickly replaces its predecessor (18). In humans, the need to escape preexisting immunity exerts positive selection pressure on changes in amino acids comprising the antigenic sites of the surface glycoproteins, HA and (NA) (18). The process of progressive change in the antigenic properties of the virus is called antigenic drift and results in the emergence of an antigenically distinct variant strain every 2-3 years (18). In the pandemic influenza, one or both of the viral surface proteins are replaced with proteins to which the human population has no preexisting immunity (18). The virus then spreads explosively, producing symptomatic infection in up to one third of most populations (18). This happened with the pandemics of 1918, 1957, and 1968 with the 1918 influenza pandemic being the most recognized and pathogenic.

Although most deaths from the 1918 pandemic were the consequence of secondary bacterial pneumonias (there were no antibiotics available in 1918), a subset died in just a

few days with massive pulmonary hemorrhage or edema. Clinical symptoms and pathological findings during the 1918 influenza pandemic were predominantly respiratory ones. Necrotic lesions in systemic organs like those seen in virulent avian influenza infections were not observed. Gastrointestinal symptoms and impaired hepatic and renal function also were described.



## Hemagglutinin (HA)

Hemagglutinin (HA) is a major influenza surface glycoprotein that is involved in binding of the host cell during infection. There are 16 different HA subtypes, all of which wild aquatic birds are the reservoirs (4). Of the 16 HA subtypes only H1, H2 and H3 have caused human disease and death (4). Hemagglutinin was so named because it is the protein responsible for the ability of influenza virus to agglutinate red blood cells. HA is now recognized as the major virulence factor associated with the influenza virus.

HA is translated as a single protein, HA<sub>0</sub>, and expressed as a trimer (5). In order for influenza to infect the host cell, HA<sub>0</sub> must be activated by cleavage by a trypsin-like serine endoprotease at a specific site, normally coded for by a single basic amino acid (usually arginine) between the HA<sub>1</sub> and HA<sub>2</sub> domains of the protein (5). Highly basic amino acids in the HA binding site are associated with pathogenesis in chickens and mice (6-8). Interestingly, the 1918 pandemic influenza virus does not have multiple basic amino acids at the cleavage site (9). In mammals, the suspected protease in the respiratory tract responsible for HA activation is tryptase Clara, a serine protease produced by non-ciliated Clara cells of the bronchial and bronchiolar epithelia (5). After cleavage, the two disulfide-bonded protein domains produce the mature form of the protein subunits as a prerequisite for the conformational change necessary for fusion and hence viral infectivity (5). The antigenic and receptor-binding sites of the HA<sub>1</sub> domain represent the most variable portion of the influenza genome (10).

Human influenza viruses attach to host cells via an N-acetylsialic acid attached to galactose with an  $\alpha$ -2,6 linkage, whereas avian influenza viruses mostly bind to N-

acetylsialic acid attached to galactose with an  $\alpha$ -2,3 linkage (11). This binding specificity of the hemagglutinin protein determines the host range of a given influenza virus (11). Human tracheal epithelium contains mostly  $\alpha$ -2,6 receptors whereas duck gut epithelium contains mostly  $\alpha$ -2,3 receptors (11). However, chicken lung and intestinal epithelial cells contain both  $\alpha$ -2,6 and  $\alpha$ -2,3 receptors (11). The lack of human-to-human transmission of avian influenza H5N1 viruses is believed to be due to their  $\alpha$ -2,3 sialic acid receptor binding preference (12). The three influenza pandemic viruses of the last century, occurring in 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2), each possessed an HA with a human  $\alpha$ -2,6 sialic acid binding preference (12).

Tumpey et al. (12) have shown that amino acids 190 and 225 in the 1918 HA determined its binding specificity. Three influenza viruses were used in this study with differing specificities. A/South Carolina/1/18 (SC18) was specific for  $\alpha$ -2,6 sialic acid. A single amino acid substitution in SC18 from aspartate to glycine at position 225 gave it a mixed  $\alpha$ -2,3/ $\alpha$ -2,6 sialic acid preference designated as NY18 (12). The virus with  $\alpha$ -2,3 sialic acid preference (AV18) was made by substituting aspartate for glutamate at position 190 in NY18 (12). Transmission of the 1918 pandemic virus was abolished when the binding specificity was changed from  $\alpha$ -2,6 (human) to  $\alpha$ -2,3 (avian) preference (12). Ferrets inoculated with NY18 and AV18 showed severe illness and death but failed to transmit the virus ferret-to-ferret (12). The AV18 virus also replicated in the upper respiratory tract as efficiently as the parental SC18 virus, but it failed to transmit to contact ferrets (12). These same amino acid changes do not confer  $\alpha$ -2,6 preference on H5N1 avian influenza viruses. It is therefore likely that different avian HA subtypes have different

structural requirements to confer receptor specificity (12). It is not known which amino acid changes will need to occur in order for H5N1 to gain  $\alpha$ -2,6 specificity (12).

Since the HA protein is so important in determining host specificity it has been an important focus in determining its role in the high pathogenicity of the 1918 pandemic influenza strain. Both the 1918 HA and 1918 polymerase genes have been shown to be essential for maximal virus replication and optimal virulence (13 - 15). In order to test what effect the HA protein had on the pathogenicity of the 1918 pandemic virus Kobasa et al. (15) placed the 1918 HA in the background of three different influenza strains, A/WSN/33 (WSN), A/Kawasaki/173/2001 (K173), and A/Memphis/8/88 (M88). Mice were inoculated intranasally with each of the three recombinant viruses. The parental K173 and M88 caused no discernible morbidity in mice whereas the K173 and M88 strains bearing the HA from 1918 caused a lethal infection in mice (15). WSN with and without the HA from 1918 were pathogenic. Since WSN is already highly pathogenic in mice it could not be determined what the contribution of the 1918 HA had on the pathogenicity of the recombinant virus (15). Infection with viruses possessing HA from 1918 was characterized by massive recruitment of polymorphonuclear cells (mainly neutrophils) accompanied by intra-alveolar haemorrhage (15). 1918 HA was found to be the primary determinant of enhanced pathogenicity in the recombinant viruses (15).

In mammals, like humans and swine, influenza replication is limited to epithelial cells of the upper and lower respiratory tract. This tissue tropism is controlled to some extent by the limited expression of the appropriate protease for viral activation of the HA protein (5). Avian strains have been found with insertion mutations at the HA cleavage site which allows HA to be cleaved by ubiquitously expressed proteases (5). This allows the

virus to be able to replicate throughout the birds body (5). This mutation inserts an additional amino acid with a minimal motif of R/L-X-R/L-R (5). This has only been found in avian H5 and H7 subtypes recently (5). In 1997 16 people in Hong Kong were infected with an avian H5N1 influenza virus (5). Although the HA cleavage site mutation had not been found previously in humans, an influenza strain with the ability to replicate outside its normal host cells was described 50 years ago. WSN/33 was produced in 1940 by forcing the parent strain, WS/33, to replicate in mouse brain (16) to develop an animal model for the observed neurologic complications associated with the 1918 influenza. The strain was passaged extensively in ferrets, in chicken eggs, in mouse lung, and finally in mouse brain. Although this strain was believed initially to be specifically pneumotropic and neurotropic, producing a lethal encephalitis in mice, it recently has been shown to be pantropic or capable of systemic infection in mice (17). It was found that WSN/33 had its HA cleaved by serum plasmin found in the brain. Schulman and Palese (33) showed that the NA protein of WSN/33 was a necessary component for HA cleavage, and in 1993, Li et al. (34) found that the NA of WSN/33 lacked a crucial glycosylation site at residue 130 that is conserved in other influenza NA's. The N146R change in WSN/33 alters the N-X-S/T motif necessary for posttranslational glycosylation of NA (15). Plasminogen binds specifically to the NA of WSN/33. In so doing, the WSN/33 NA sequesters plasminogen on the cell surface so that it can be activated. Once activated, plasmin, also a serine protease, recognizes the single arginine motif at the cleavage site and cleaves HA<sub>0</sub> into HA<sub>1</sub> and HA<sub>2</sub>. WSN/33 neurovirulence also required the matrix (M) and nonstructural (NS) segments in addition to NA. The M and NS segments seemed to act as accessory virulence factors to enable efficient viral replication. The biological behavior of

neurovirulence is polygenic and cannot be fully explained by potentially pantropic HA cleavage alone.

## Neuraminidase (NA)

Neuraminidase (NA), like hemagglutinin, is a surface-expressed glycoprotein. Along with HA, NA plays an important role in virulence, host specificity and the human immune response (10). NA cleaves the terminal sialic acid residues that are receptors for the HA protein (10). Removal of sialic acids from the surface of infected cells and from newly formed viruses prevents the budding viruses from clumping to each other or the cell surface (19). The ability to cleave sialic acid is also thought to help the virus penetrate mucus (20). There are nine subtypes of NA that have been identified (10). The active site consists of 15 charged amino acids in a pocket on the surface. These amino acids are conserved in all influenza A viruses (21). NA demonstrates more genetic variability than other influenza genes (10). There are at least two antigenic sites in NA (22). Mutations in these amino acids can allow the virus to escape previous immunity (22). Shift in NA is not critical in the initiation of a pandemic, as the pandemic strain of 1968 retained the previously circulating N2 (10). However, widespread immunity to N2 is thought to have lessened the severity of the 1968 pandemic (23, 24).

Phylogenetic analyses revealed that the 1918 NA sequences are most closely related to avian isolates, but also suggest that the 1918 sequences share enough characteristics with mammalian isolates to distinguish them from the avian clade (10). The results of NA analysis from the Spanish 1918 influenza virus, A/Brevig Mission/1/18 (Brevig/18), sequence is intermediate between avian and mammalian sequences, and therefore are consistent with the idea that pandemic viruses acquire their surface proteins directly (with little modification) from avian viruses. There are 22 homologous amino acids in avian

N1's (21). Fifteen of these 22 amino acids show variation in human N1's (10). The sequence of the Brevig/18 NA matches 14 of the 15 avian consensus sites (10).

Normally, neuraminidase activates latent TGF- $\beta$ , a potent anti-inflammatory cytokine that activates monocytes (4). During infection in mice with different Hong Kong H5N1 strains it was noted that there was a decrease in TGF- $\beta$  activation (4). Thus, NA may help contribute to the severe pathology of H5N1 infections in mice by decreasing TGF- $\beta$  activation in the infected host (4).

## Non-Structural Protein (NS)

The non-structural protein (NS) of influenza is so named due to the fact that it is not incorporated into the influenza particle. Once the virus has infected a cell it will express the NS protein. The NS protein is cleaved into two separate proteins, NS1 and NEP (nuclear export protein). NEP is believed to aid in the export of viral nucleic acid across the nuclear envelope of the host cell. NS1 is known to attenuate the host response mediated by  $\alpha$  and  $\beta$  interferons (25). Sang Heui Seo et al. found that H5N1 Hong Kong strains were not inhibited by IFN- $\alpha$ , IFN- $\gamma$  or TNF- $\alpha$  when added to St. Jude porcine lung (SJPL) epithelial cells (26). When they added the NS gene of the H5N1 Hong Kong strain A/HK/156/97 to A/PR/8/34 (H1N1) with reverse genetics they found that the recombinant virus inherited the same cytokine resistance that the H5N1 strain had, whereas the parent A/PR/8/34 strain was susceptible to the cytokines (26). This study showed that it was the NS gene of H5N1 avian influenza that was imparting cytokine resistance to the virus. They also showed that the change from aspartic acid to glutamic acid at position 92 of NS1 was crucial for pathogenesis (26). Post-mortem reports of two human deaths caused by H5N1/97 viruses indicated that the chief cause of death was due to hypercytokinemia and resultant hemophagocytic syndrome due to high levels of cytokines (26). This was also seen in non-human primates challenged with the wild-type 1918 pandemic influenza virus (35).



## Reverse Genetics

In contrast to positive-sensed RNA viruses, the naked genomic RNA of a negative-sensed RNA virus is not able to initiate infection when expressed or transfected into a permissive cell line (27). The minimal infectious particle of negative-sensed RNA is the transcriptionally active ribonucleoprotein (RNP). This complex is composed of genomic viral RNA (vRNA) along with viral nucleoprotein (NP) and the RNA-dependant RNA polymerase (PA, PB1 and PB2) (27). The NP, PB1, PB2, and PA proteins are the only proteins required for replication and transcription of the viral RNP's *in vivo* (29). These genomic RNPs are templates for transcription to produce naked mRNAs as well as for replication in order to form negative-stranded RNA for encapsulation (36).

Enami et al pioneered the reverse-genetics, helper virus-dependent system for influenza A virus (30). The influenza ribonucleoprotein (RNP) complex is generated by *in vitro* vRNA synthesis in the presence of purified polymerase and NP proteins and then used to transfect eukaryotic cells (30). Subsequent infection with influenza A helper virus results in the generation of viruses possessing a gene derived from cloned cDNA (30).

Whereas Enami et al (30) used *in vitro* techniques in order to produce transfectant virus, Neumann et al. (31) used *in vivo* synthesis of vRNA by RNA polymerase I (polI), a cellular enzyme that transcribes ribosomal RNA that lacks both a 5' cap and a 3' poly(A) tail (31). Transfection of cells with a plasmid containing cloned influenza virus cDNAs, flanked by RNA polymerase I promoter and terminator sequences, followed by influenza virus infection, led to the production of transfectant viruses (31).

Pleschka et al (27) designed a plasmid-based reverse genetics system to make RNP's without the need of a recombinant helper virus or purified proteins. Four plasmids encoding for the P proteins (PB1, PB2 and PA) and NP, under the control of the hydroxymethylglutaryl-coenzyme A reductase (HMG) promoter, were used to make their respective proteins. A fifth plasmid encodes the chloramphenicol acetyltransferase (CAT) open reading frame in a negative-sense flanked by the non-coding regions of the NS gene of influenza A/WSN/33. A truncated human polII polymerase promoter (-250 to -1) was fused directly to the end of the viral cDNA in order to ensure the correct 5' end of the vRNA. This promoter was selected by Pleschka et al. due to the success of Neumann et al. of a similarly truncated murine polII promoter to drive the expression of influenza virus model RNA in virus-infected cells (31). The hepatitis delta virus genomic ribozyme was added to the 3' end in order to ensure that the 3' end was correct (27). These plasmids were transfected into 293T cells which resulted in CAT protein expression. This indicates that a negative-sense RNA can be reconstituted intracellularly into functional RNP's. These intracellularly reconstituted RNP's were packed into progeny influenza viruses when the plasmid-transfected cells were infected with influenza WSN virus.

All these methods required that the transfectants be selected from a vast background of helper viruses, which requires a strong selection system and complicates the generation of growth-defective viruses. Neumann et al then designed a system in which influenza virus was made completely from cloned cDNAs (32). This reverse-genetics approach is highly efficient and can be used to introduce mutations into any gene segment and to develop influenza virus-based gene delivery systems. All eight cDNAs representing the

eight viral RNA segments were placed under the control of the human RNA polymerase I promoter and mouse RNA polymerase I terminator in the pCAGGS plasmid (32). These plasmids were then transfected into 293T cells along with protein expression plasmids expressing influenza PB1, PB2, PA and NP. vRNAs and RNPs were successfully produced along with infectious viral particles (32). Producing influenza virus from cloned cDNAs allows for the mixing of RNA from different influenza strains. This allows for the testing of specific genes on the pathogenicity and infectivity of influenza viruses.

## **Mice Influenza Model**

Mice have been used as model hosts in a variety of viral infections. After human infections with the highly-pathogenic H5N1 viruses in 1997 occurred, scientists started to study the virus. No suitable host model had been established for highly-pathogenic influenza viruses. The BALB/c mouse was found to be a suitable animal model for the study of highly-pathogenic H5N1 human virus pathogenesis and immunity (38). The H5N1 virus caused lethal infection and replicated in lungs of mice with no prior adaptation, something normally required (38). Tumpey et al. showed that there was a decrease in peripheral blood and tissue lymphocytes and aberrant cytokine and chemokines production in mice when challenged with the H5N1 virus (39). This demonstrated that the BALB/c mouse was a good model for the immune response to H5N1. They also showed that there was an increase in apoptotic cells in the spleen and lung tissue, a possible cause of lymphocyte death (39).

## **Study Objectives**

This study looked at the pathogenic effects of the 1918 pandemic influenza viral HA, NA and NS genes in the background of the low-pathogenic TX91 influenza virus. The highly-pathogenic H5N1 A/Vietnam strain was used as a “positive” control for highly pathogenic viral pathogenesis. BALB/c mice were utilized and lung titers and body weight were determined.

## Materials and Methods

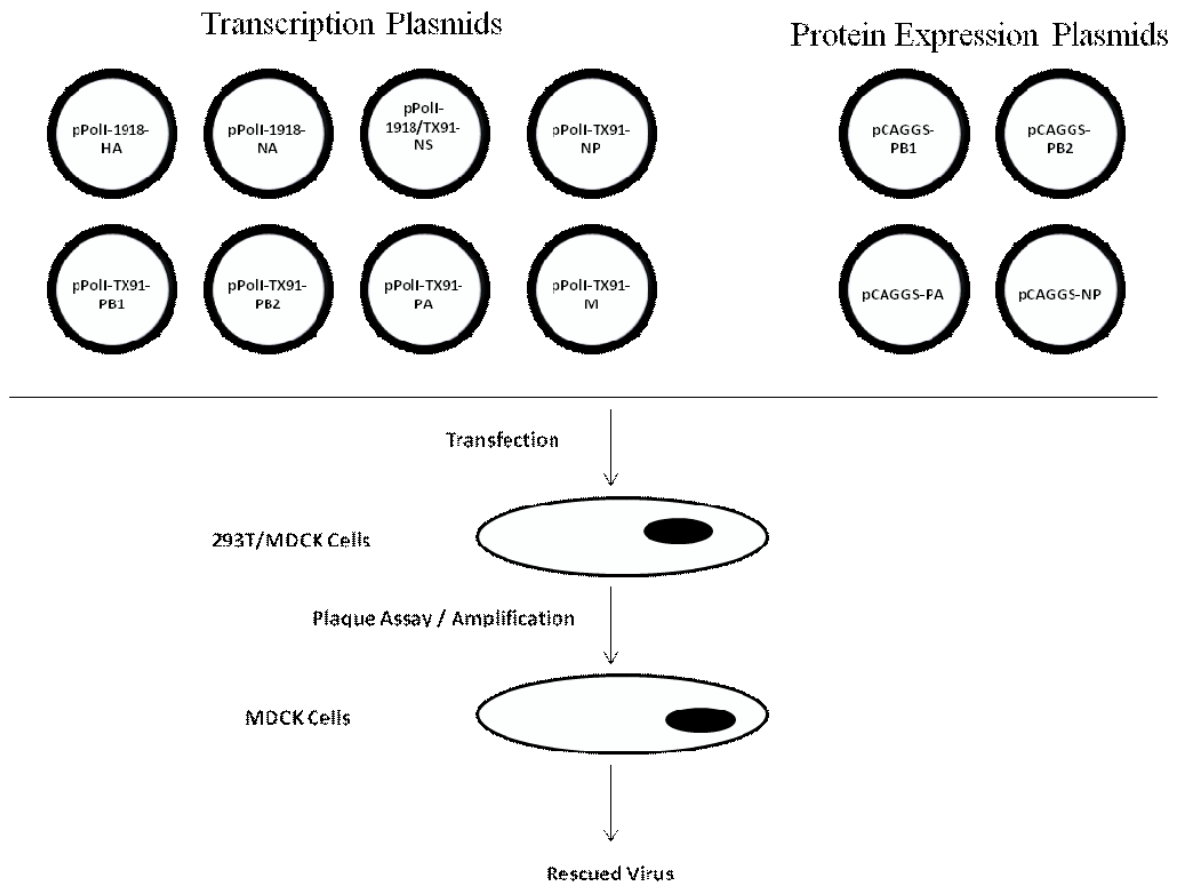
**Cells.** 293T human embryonic kidney cells and Madin–Darby canine kidney cells (MDCK) were maintained in DMEM supplemented with 10% FBS and in EMEM containing 10% FBS, respectively. All cells were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

**Generation of Partially-Reconstructed Influenza.** Influenza particles containing RNA segments from the 1918 pandemic influenza virus was constructed in the background of the innocuous influenza strain A/Texas/36/91 (TX91). In one partially reconstructed 1918 virus we placed the 1918 HA (from A/South Carolina/1/18) and NA (from A/Brevig Mission/1/18) cDNAs in the background of TX91. In another we placed the 1918 HA (from A/South Carolina/1/18), NA (from A/Brevig Mission/1/18) and NS (from A/Brevig Mission/1/18) cDNAs in the background of TX91. All three of these proteins have been determined to be influenza virulence factors.

The following plasmids were used in this study: pCAGGS-PB1, pCAGGS-PB2, pCAGGS-PA, and pCAGGS-NP (protein expression plasmids) along with pPolI-TX91-PB1, pPolI-TX91-PB2, pPolI-TX91-PA, pPolI-TX91-NP, pPolI-TX91-M, pPolI-1918-HA, pPolI-1918-NA and pPolI-1918-NS (RNA polymerase I plasmids containing viral cDNAs) (Figure 1). All plasmids were obtained from the laboratory of Dr. Garcia-Sastre, Mount Sinai Medical Center. The eight transcription plasmids expressing the individual vRNA genes were constructed by polymerase chain reaction (PCR), using overlapping deoxyoligonucleotides for the genes. The coding regions are according to the open reading frame sequences. The viral genomic sequences were cloned into the

pPoll.SapI.Rib vector. This vector contains the human RNA polymerase I promoter (Pol-I) and the hepatitis  $\delta$  ribozyme (R) sequence and originated from Dr. Ervin Fodor at the University of Oxford.

Figure 1. Schematic representation of the plasmid-based rescue system for the influenza viruses.



Each transfection contained vRNA expression plasmids for the TX91 PA, PB1, PB2, NP, and M segments, the appropriate vRNA expression plasmid for the 1918 HA, NA and NS segments, and the protein expression plasmids pCAGGS-PA, -PB1, -PB2 and -NP.

Two control plasmids were also used. The pCAGGS-PB1/PB2/PA/NP/GFP control plasmid monitored polymerase complex activity via GFP expression. The pCAGGS-GFP control plasmid monitored transfection efficiency via GFP expression.

The 1918 HA gene sequence used in this study was obtained from formalin-fixed, paraffin-embedded right lung tissue from a 21-year-old male stationed at Ft. Jackson, South Carolina (A/South Carolina/1/18) (2).

The 1918 NA and NS gene sequences used in this study came from a 1918 influenza virus isolated from an Inuit woman exhumed in permafrost in Brevig Mission in Alaska (10). History shows that the 1918 pandemic virus swept through the village of Brevig Mission in 5 days, leaving 72 dead (1).

The protein expression plasmids were derived by transferring the ORFs of the WSN PA, PB1, PB2, and NP into the pCAGGS expression plasmid.

In order to make 1918 HA/NA:TX91 recombinant influenza virus, 293T/MDCK cell mixture (7:10 ratio, respectively) were transfected with 1 $\mu$ g of pPolII plasmids containing the HA and NA genes from 1918 and the remaining six genes from TX91 along with 1 $\mu$ g pCAGGS plasmids containing the cDNA coding for NP, PB1, PB2 and PA proteins using Lipofectamine 2000 (Invitrogen, Carlsbad, California).



In order to make 1918 HA/NA/NS:TX91 recombinant influenza virus, 293T/MDCK cell mixture were transfected with 1µg of pPolI plasmids containing the HA, NA and NS genes from 1918 and the remaining five genes from TX91 along with 1µg pCAGGS plasmids containing the cDNA coding for NP, PB1, PB2 and PA proteins using Lipofectamine 2000 (Invitrogen, Carlsbad, California).

After 18-24 hours, the media was aspirated and replaced with 1x MEM containing 1µg/ml TPCK-trypsin. Plates of cells were continued to be incubated for an additional 48 hours at 37°C and 5% CO<sub>2</sub>.

Recombinant virus was plaque-purified approximately 72 hours post-transfection. Briefly, 500µl of transfected supernatant was added to a well of a 6-well plate of MDCK cells and incubated 1 hour at room temperature in order to allow the virus to adsorb to the cells. Cells were then overlaid with 3mls of 1.8% agarose and incubated at 37°C and 5% CO<sub>2</sub> in order to allow virus growth. Approximately 72 hours later plaques were picked and placed into tubes containing 1x MEM. The picked plaques were then expanded on confluent MDCK cells.

RNA was purified with TriPure (Roche Applied Science, Indianapolis, IN) and sent to the Mount Sinai Medical Center for sequencing in order to verify no mutations were present in the viral genomes of the reconstructed 1918 viruses.

Identification of all genes in the reconstructed viruses was confirmed by RT-PCR and subsequent sequencing.

Titers of the partially-reconstructed recombinant 1918 viruses were determined by TCID<sub>50</sub> analysis.

**Viruses.** Stocks of 1918 HA/NA:TX91 and 1918 HA/NA/NS:TX91 were propagated on Madin-Darby canine kidney cells (MDCK). The highly pathogenic A/Vietnam/1203/04 virus (A/Vietnam) was propagated in the allantoic cavity of 10 day old hen eggs at 37°C. The allantoic fluid from infected eggs was harvested and stored at -70°C. The A/Vietnam virus used for challenge experiments was passed a single time in embryonated eggs prior to use. A/Texas/36/91 (TX91) was obtained from the laboratory of Dr. Adolfo Garcia-Sastre at the Mount Sinai Medical Center (New York, New York). Virus titers were determined by plaque assays in MDCK cells and are expressed as plaque forming units per milliliter (PFU/mL). All viruses were stored at -70°C until use.

**Lung processing.** Necropsies were carried out according to a standard protocol. Samples for virological examination were stored at  $\leq -70^{\circ}\text{C}$ . Lungs from 9 mice from each group at each time point were homogenized in a single 50 cc tube containing minimal essential medium (MEM) containing 100 IU of penicillin per ml, 100 mg of streptomycin per ml, 4% bovine serum albumin (fraction V; Gibco-BRL), and 4 mg of trypsin (Gibco-BRL) (infection medium) using Potter tissue grinders. Lungs were pooled into groups of 3 so that  $n=3$  for each group at each time point. The supernatant was centrifuged at 700 x g for 5 minutes at 4°C. The clarified supernatant was transferred to cryovials and stored at  $\leq -70^{\circ}\text{C}$  for viral load analysis.

**Mice.** Female BALB/c mice (5-6 weeks of age) were purchased from Charles Rivers Laboratories (Wilmington, MA). Mice were housed in polycarbonate filter cages inside a Biosafety level 3 enhanced facility (BSL-3+). Food and water were provided ad libitum.

**Lethality.** To confirm lethality of the partially-reconstructed 1918 HA/NA:TX91 and 1918 HA/NA/NS:TX91 virus stocks, groups of 3-7 mice were anesthetized with ketamine and xylazine and challenged via the intranasal route (IN) in a total volume of 50 microliters with approximately  $1 \times 10^6$  PFU of the 1918 HA/NA:TX91, or 1918 HA/NA/NS:TX91 virus. For purposes of comparison, groups of mice were also challenged with  $2 \times 10^4$  PFU of A/Vietnam or vehicle controls (uninfected MDCK cell culture supernatant or alloantoic fluid). Body weights and clinical observations were recorded daily over a 12 day period post-challenge. Animals surviving challenge were euthanized. The experiment was repeated for the 1918 HA/NA:TX91 and TX91 viruses using 4 serial 10-fold or 3 serial 3-fold dilutions of virus, respectively, in phosphate buffered saline (4-6 mice for each dose/dilution).

**Pathogenicity.** Mice were infected with 50 microliters of either A/Vietnam ( $2.0 \times 10^4$  PFU), TX91 ( $1.1 \times 10^6$  PFU), 1918 HA/NA:TX91 ( $1.7 \times 10^6$  PFU), 1918 HA/NA/NS:TX91 ( $1.4 \times 10^6$  PFU) or vehicle controls (uninfected MDCK cell culture supernatant or alloantoic fluid) via the intranasal route (Table 1). An additional 5 naïve mice were anesthetized with ketamine and xylazine and a terminal blood sample taken by heart stick one day prior to the start of the experiment. These mice served as naïve controls. At approximately 24, 72 and 120 hours following infection 9 mice per group were sacrificed for collection of lungs. Lungs were pooled from 3 mice per time point per group in order to ensure sufficient sample volume for assays.

Table 1. Stock influenza viral titers and challenge doses.

<b>Group</b>	<b>Abbreviation</b>	<b>Titer<sup>1</sup> (PFU/mL)</b>	<b>Challenge dose (PFU)</b>
HA 1918 / NA 1918 / 6 genes from A/Texas/36/91	1918 HA/NA:TX91	$3.4 \times 10^7$	$1.7 \times 10^6$
HA 1918 / NA 1918 / NS 1918 / 5 genes from A/Texas/36/91	1918 HA/NA/NS:TX91	$2.7 \times 10^7$	$1.4 \times 10^6$
A/Vietnam/1203/04 (H5N1)	A/Vietnam	$1.5 \times 10^8$	$2.0 \times 10^4$
A/Texas/36/91 (H1N1)	TX91	$3.0 \times 10^8$	$1.1 \times 10^6$
Cell culture vehicle control	CC	0	NA
Allantoic culture vehicle control	AC	0	NA

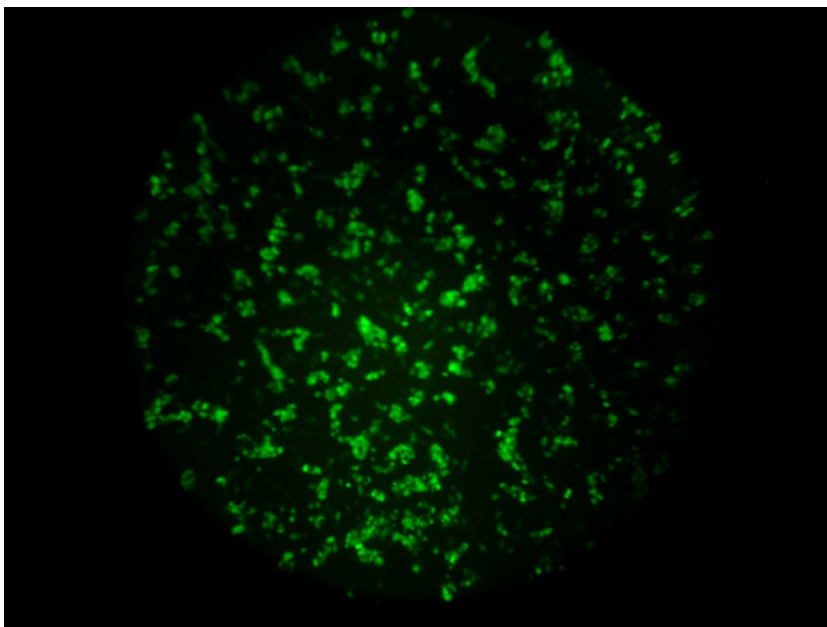
<sup>1</sup> Titers calculated using the Spearman Kärber method from titrations in MDCK cells.

**TCID<sub>50</sub>.** In order to assess the viral load in the lungs Tissue Culture Infectious Dose 50 (TCID<sub>50</sub>) assay was used. Lung homogenates were added to 96-well plates seeded with MDCK cells. Three samples were assayed per group per time point. Samples were diluted 1:10 initial dilution and then nine times at 1:5 for a total of ten dilutions. Five replicates of each dilution were plated. Samples were incubated in a humidified incubator at 37°C and 5% CO<sub>2</sub> for approximately 72 hours. Results were calculated using Spearman Kärber method.

## Results

**Reconstruction.** Genes encoding the 1918 pandemic influenza virus were reconstructed from deoxyoligonucleotides and corresponded to the reported 1918 coding sequences (2, 10, 37). Partially-reconstructed 1918 influenza viruses were produced by transfecting the appropriate plasmids from the 1918 pandemic virus and the Texas/91 virus into a 293T/MDCK cell culture mixture. Control plasmids containing a GFP marker were used in order to verify that the viral polymerase was produced and functional for viral RNA replication. Approximately 24 hours post-transfection the control transfections were viewed under a fluorescence microscope for GFP production. It was verified that the transfections were successful and that the influenza RNA-dependant RNA polymerase was being produced and was functional (Figure 2).

Figure 2. 293T/MDCK cells transfected with pCAGGS-PB1/PB2/PA/NP/GFP



Appropriate 1918 HA/NA:TX91 and 1918 HA/NA/NS:TX91 clones were chosen based on genomic RNA yield, sequence analysis, hemagglutination assay results, stock viral concentration and lethality in mice.

**Infectivity.** The HA, NA and NS genes of the 1918 pandemic influenza virus rescued in the genetic background of TX91 virus had high infectivity titers in MDCK cells similar to those of the wild-type TX91 virus. See Table 1.

**Lethality Determination.** To determine the lethality of the 1918 HA/NA:TX91 construct, serial 10-fold dilutions of virus were inoculated into groups of four mice ( $1.7 \times 10^6$  -  $1.7 \times 10^3$  PFUs; Figure 3). The results demonstrate that the virus was 100% lethal at  $1.7 \times 10^6$  PFU and  $1.7 \times 10^5$  PFU. The 1918 HA/NA:TX91 construct was also lethal at  $1.7 \times 10^4$  PFU, although not 100%. By comparison, challenge with undiluted ( $1 \times 10^6$  PFU) 1918 HA/NA/NS:TX91 resulted in only approximately 50% mortality (Figure 4). The parent TX91 virus was also tested and was found not to be lethal.

Figure 3. Lethality determination of 1918 HA/NA:TX91. BALB/c mice were inoculated intranasally with serial dilutions of 1918 HA/NA:TX91 from  $1.7 \times 10^6$  PFU to  $1.7 \times 10^3$  PFU and monitored for 14 days for survival.

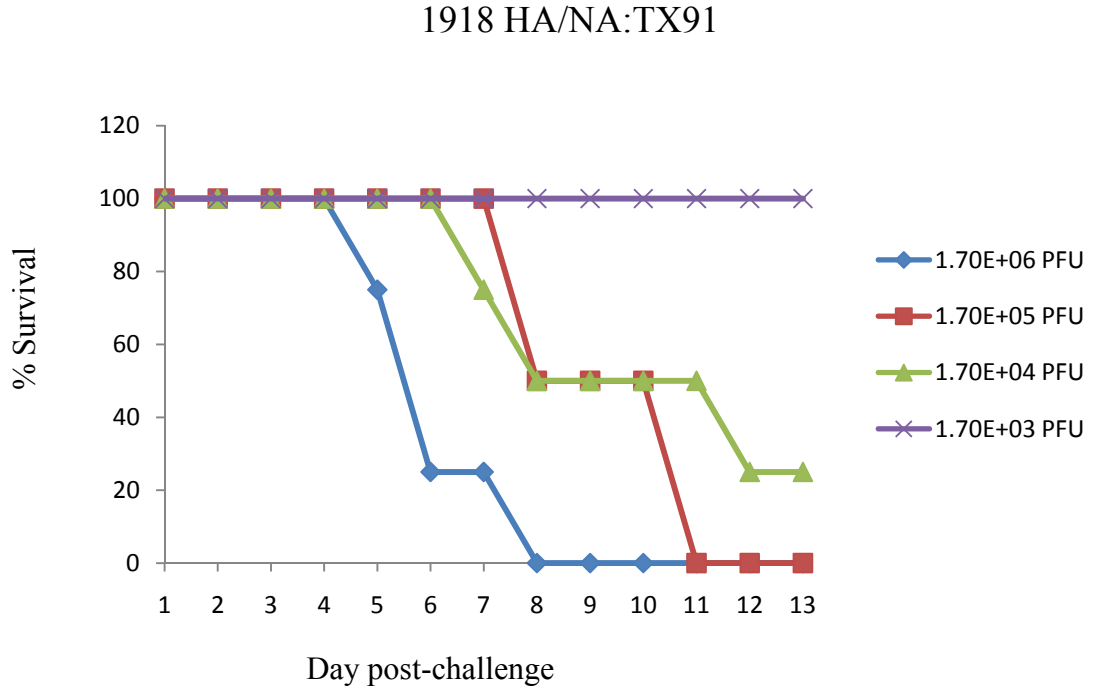
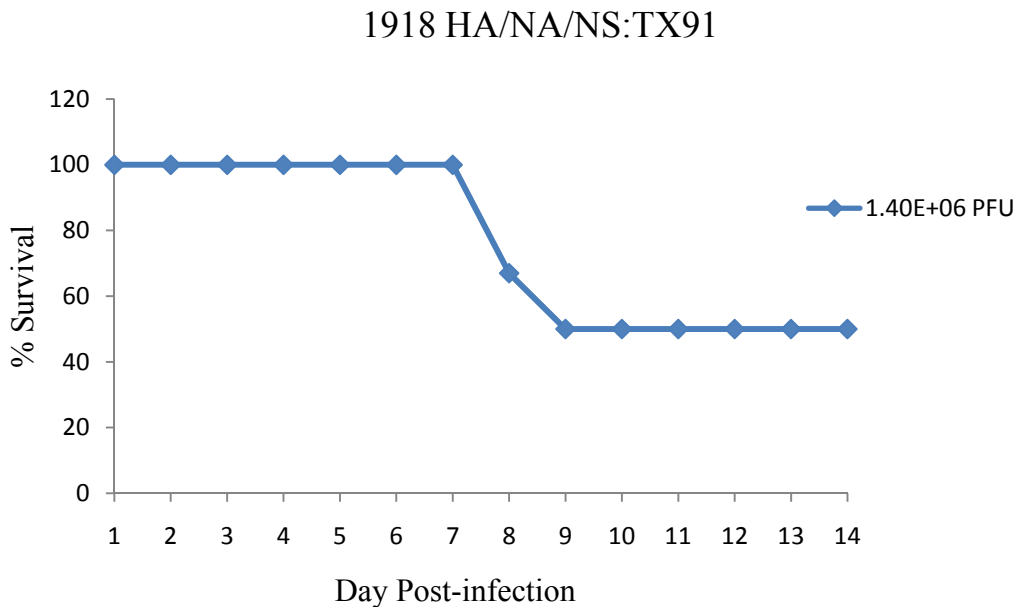


Figure 4. Lethality determination of 1918 HA/NA/NS:TX91. BALB/c mice were inoculated intranasally with  $1.4 \times 10^6$  PFU of 1918 HA/NA/NS:TX91 and monitored for 14 days for survival.



**Pathogenicity.** To better understand the differences in pathogenicity among the recombinant 1918 viruses, we examined the mortality time course post-challenge (Figure 5) and percent weight loss (Figure 6) over the course of 14 days, and viral replication in the lungs at 24, 72 and 120 hours post-challenge (p.c.) (Figure 7).

Figure 5. Time course of mortality following challenge. BALB/c mice were challenged with  $1.7 \times 10^6$  PFU (1918 HA/NA:TX91),  $1.4 \times 10^6$  PFU (1918 HA/NA/NS:TX91),  $1.1 \times 10^6$  PFU (TX91),  $2 \times 10^4$  PFU (A/Vietnam), and 50 $\mu$ l cell culture and allantoic fluid controls.

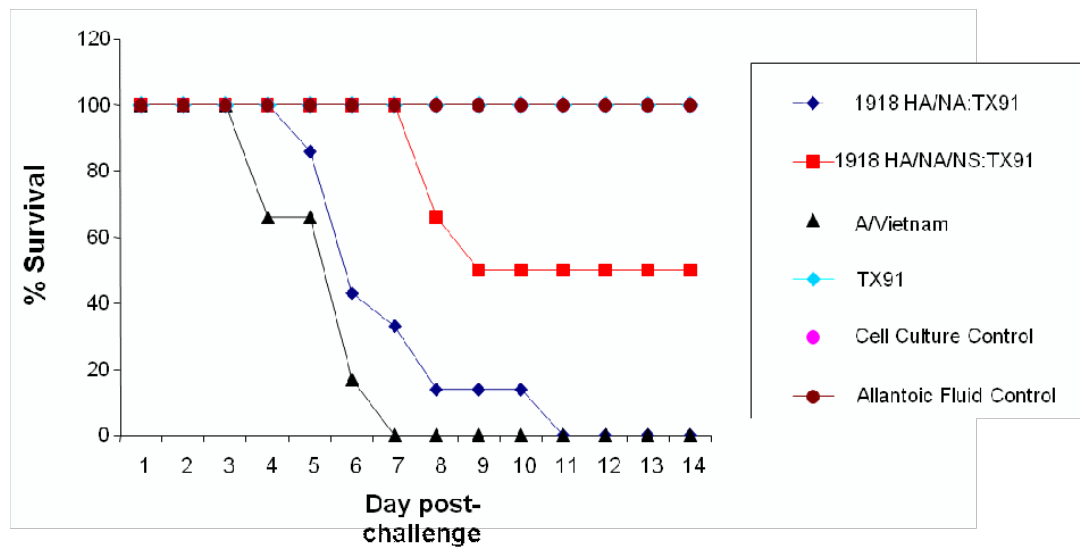




Figure 6. Change in body weight following challenge. BALB/c mice were challenged with  $1.7 \times 10^6$  PFU (1918 HA/NA:TX91),  $1.4 \times 10^6$  PFU (1918 HA/NA/NS:TX91),  $1.1 \times 10^6$  PFU (TX91),  $2 \times 10^4$  PFU (A/Vietnam), and 50 $\mu$ l cell culture and allantoic fluid controls

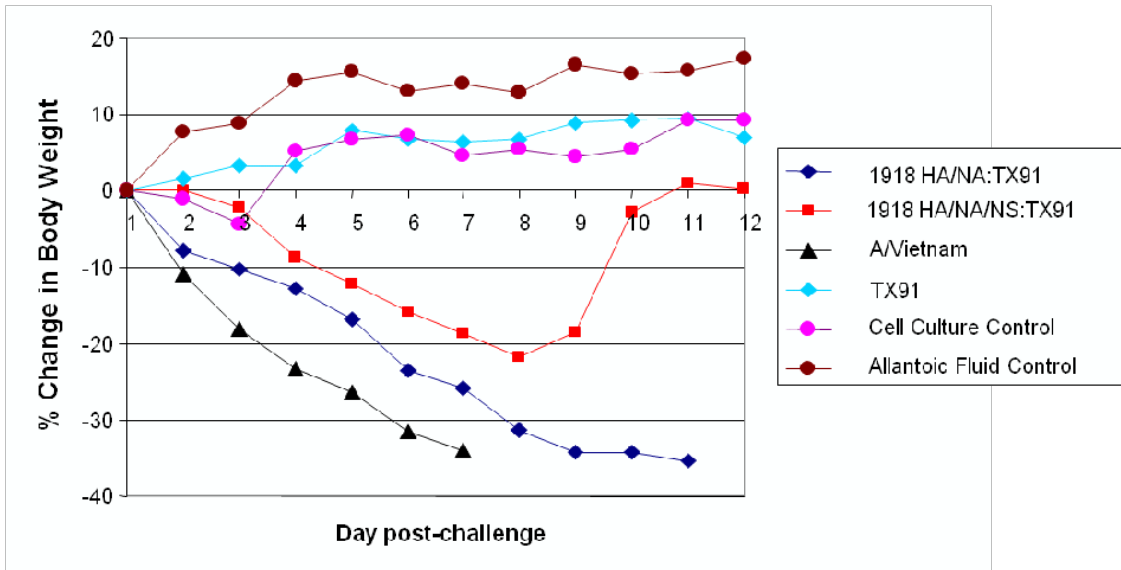
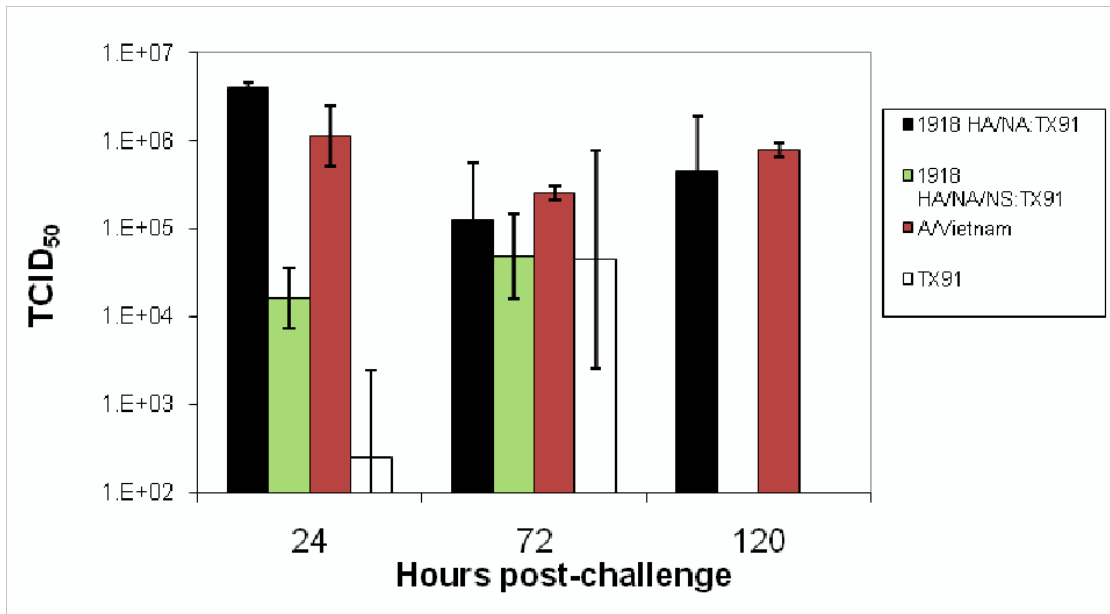


Figure 7. Mean viral lung titers (Tissue Culture Infectious Dose<sub>50</sub>) in BALB/c mice. Mice were challenged with  $1.7 \times 10^6$  PFU (1918 HA/NA:TX91),  $1.4 \times 10^6$  PFU (1918 HA/NA/NS:TX91),  $1.1 \times 10^6$  PFU (TX91), and  $2 \times 10^4$  PFU (A/Vietnam).



The A/Vietnam virus was highly pathogenic in the BALB/c mouse. Substantial weight loss was observed by day 5 post-challenge (31%) and all animals succumbed prior to day 7 (Figure 6). Similarly, mice challenged with the 1918 HA/NA:TX91 virus lost approximately 23% body weight by day 5 post-challenge and all mice succumbed prior to day 11. The 1918 HA/NA/NS:TX91 virus was less pathogenic in that challenge with approximately  $1 \times 10^6$  PFU resulted in 15-16% weight loss by day 5 and 50% mortality. Interestingly, results for mice challenged with the parent  $1 \times 10^6$  PFU of TX91 were similar to those obtained for vehicle controls. Mice in the TX91 and vehicle control groups gained weight following challenge and all animals survived.

All mice challenged with cell culture control, allantoic fluid control and TX91 survived challenge (Figure 5). Only about 50% of mice challenged with 1918 HA/NA/NS:TX91 survived challenge through day 14. These were the same results seen in the LD<sub>50</sub> study at the same dose. Mice challenged with A/Vietnam and 1918 HA/NA:TX91 started dying by day 4 post-challenge. By day 7 p.c. all mice challenged with A/Vietnam had died and by day 11 p.c. all mice challenged with 1918 HA/NA:TX91 had died. We can see that the 1918 HA/NA:TX91 recombinant virus is as lethal as A/Vietnam but with slower kinetics. The 1918 HA/NA/NS:TX91 virus was more lethal than the parent TX91 virus, but not as lethal as the 1918 HA/NA:TX91 recombinant virus or the highly-pathogenic A/Vietnam influenza virus strain. It appears that the addition of the 1918 NS gene attenuates the virus. This attenuation has been seen in previous studies in mice (37).

Influenza virus was detected in the lungs of mice challenged with A/Vietnam, TX91 and both 1918 reassortants at 24 hours (Figure 7). The highest viral titers were detected in mice at 24 hours p.c. with 1918 HA/NA:TX91 ( $3.89 \times 10^6$  TCID<sub>50</sub>/ml)(Table 2). Lung titers in these mice remained high at 72 and 120 hours p.c. ( $1.26 \times 10^5$  TCID<sub>50</sub>/ml and  $4.55 \times 10^5$  TCID<sub>50</sub>/ml, respectively). These lung titers were comparable at all time points to the highly-pathogenic A/Vietnam virus (Table 2). Lung titers in mice challenged with the 1918 HA/NA/NS:TX91 virus were lower than the 1918 HA/NA:TX91 virus at all time points. By 120 hours p.c., virus was undetectable in the lungs of mice challenged with the 1918 HA/NA/NS:TX91 virus. Lung titers in mice challenged with TX91 were lower, but comparable to mice challenged with the 1918 HA/NA/NS:TX91 virus at all time points. Vehicle control samples were uniformly negative for virus in the TCID<sub>50</sub> assay.

Table 2. Lung titers at 24, 72, and 120 hours post-challenge in BALB/c mice challenged with influenza viruses.

<b>Group</b>	<b>Time Point (hours)</b>	<b>Geometric Mean</b>	<b>95% Confidence Interval</b>
1918 HA/NA:TX91	24	$3.89 \times 10^6$	$(2.45 \times 10^6, 6.18 \times 10^6)$
	72	$1.26 \times 10^5$	$(2.87 \times 10^3, 5.49 \times 10^6)$
	120	$4.55 \times 10^5$	$(1.23 \times 10^4, 1.68 \times 10^7)$
1918 HA/NA/NS:TX91	24	$1.64 \times 10^4$	$(2.18 \times 10^3, 1.23 \times 10^5)$
	72	$4.78 \times 10^4$	$(2.88 \times 10^3, 7.94 \times 10^5)$
	120	0	(--)
A/Vietnam	24	$1.13 \times 10^6$	$(1.51 \times 10^5, 8.43 \times 10^6)$
	72	$2.52 \times 10^5$	$(1.59 \times 10^5, 3.99 \times 10^5)$
	120	$7.78 \times 10^5$	$(4.91 \times 10^5, 1.23 \times 10^6)$
TX91	24	$9.21 \times 10^2$	$(7.02 \times 10^{-3}, 1.21 \times 10^8)$
	72	$1.12 \times 10^3$	$(1.35 \times 10^2, 9.26 \times 10^3)$
	120	0	(--)
Cell Culture Control	24	0	(--)
	72	0	(--)
	120	0	(--)
Allantoic Fluid Control	24	0	(--)
	72	0	(--)
	120	0	(--)

## Statistical analysis of TCID<sub>50</sub> Data

In order to determine if the difference in lung titers amongst groups were statistically significant, an analysis of variance (ANOVA) model was performed. The p-value was < 0.5 for each time point, indicating that there was a difference between at least two of the groups (Table 3). In order to determine which groups were significantly different, Tukey's pairwise comparisons were carried out. We see from the Tukey's calculations that that all groups had a statistically significant difference in viral lung titers except between groups one and two at 24 hours post-challenge (Table 3).

A reduced data set was also created that shows the 1918 HA/NA:TX91, 1918 HA/NA/NS:TX91 and the A/Vietnam viruses as compared to the TX91 virus (Table 4). You can see from this data set that the mean titers of all three viruses were higher than the TX91 virus at all time points. In this reduced data set, groups that had reported values that were all less than the LOD at a particular time point were not included in the analysis for that time point. The complete allantoic fluid and cell culture control groups (group 5 and 6, respectively), 1918 HA/NA/NS:TX91 (group 2) on study day 5, and TX91 (group 4) on study day 5 were removed because all observations were less than the LOD.

Table 3. Summary of ANOVA results and Tukey's Pairwise.

Time Point (hours)	ANOVA Group Effect P value	Tukey *
24	<0.0001	7.77x10 <sup>5</sup> (5<1) <0.0001
		3.27x10 <sup>3</sup> (5<2) 0.0034
		2.25x10 <sup>5</sup> (6<3) <0.0001
		2.38x10 <sup>2</sup> (2<1) 0.0499
		4.22x10 <sup>3</sup> (4<1) 0.0026
		1.22x10 <sup>3</sup> (4<3) 0.0092
72	<0.0001	2.51x10 <sup>4</sup> (5<1) <0.0001
		9.57x10 <sup>3</sup> (5<2) <0.0001
		2.24x10 <sup>2</sup> (5<4) <0.0001
		5.03x10 <sup>4</sup> (6<3) <0.0001
		1.12x10 <sup>2</sup> (4<1) 0.0002
		4.28x10 <sup>1</sup> (4<2) 0.0017
		2.25x10 <sup>2</sup> (4<3) <0.0001
120	<0.0001	9.09x10 <sup>4</sup> (5<1) <0.0001
		1.56x10 <sup>5</sup> (5<3) <0.0001
		1.56x10 <sup>5</sup> (6<3) <0.0001
		9.09x10 <sup>4</sup> (2<1) <0.0001
		9.09x10 <sup>4</sup> (4<1) <0.0001
		1.56x10 <sup>5</sup> (2<3) <0.0001
		1.56x10 <sup>5</sup> (4<3) <0.0001

Group 1 = 1918 HA/NA:TX91, Group 2 = 1918 HA/NA/NS:TX91, Group 3 = A/Vietnam, Group 4 = TX91, Group 5 = Cell culture control, Group 6 = Allantoic fluid control

\* Cells contain all pairwise differences between all groups that are statistically significant at 0.05. The format within each cell is (1) the ratio of geometric means, (2) the relationship of the corresponding pair of group geometric means shown in parentheses [For example, "(1<2)" indicates that the geometric mean for group 1 was less than that for group 2], and (3) the Tukey-adjusted P-value.

Table 4. Summary of ANOVA results and Tukey's Pairwise Comparisons for reduced data set

<b>Time Point (hours)</b>	<b>Group Effect P value</b>	<b>Tukey*</b>
24	0.0105	4.22x10 <sup>3</sup> (4<1) 0.0130
		1.22x10 <sup>3</sup> (4<3) 0.0301
72	0.001	1.12x10 <sup>2</sup> (4<1) 0.0024
		4.28x10 <sup>1</sup> (4<2) 0.0097
		2.25x10 <sup>2</sup> (4<3) 0.0010
120	0.5602	

Group 1 = 1918 HA/NA:TX91, Group 2 = 1918 HA/NA/NS:TX91, Group 3 = A/Vietnam, Group 4 = TX91

Cells contain all pairwise differences between all groups that are statistically significant at 0.05. The format within each cell is (1) the ratio of geometric means, (2) the relationship of the corresponding pair of group geometric means shown in parentheses [For example, "(1<2)" indicates that the geometric mean for group 1 was less than that for group 2], and (3) the Tukey-adjusted P-value.

## Discussion

Highly pathogenic pandemic influenza viruses cause high morbidity and mortality in the human population. This study investigated the virulence factors of the 1918 pandemic influenza. The objective was to measure the change in pathogenesis of the low pathogenic A/Texas/36/91 influenza when the HA, NA, and NS genes of the 1918 pandemic influenza were added.

Tumpey et al. showed that placing the 1918 HA and NA genes together in the background of A/WSN/33 (WSN) caused lethality in mice that was equivalent to the parent WSN virus (57). However, when 1918 HA and NA were placed in the background of WSN individually it caused an attenuation of the virus (57). When Kobasa et al. (15) placed the 1918 HA gene in the background of A/Kawasaki/173/2001 (H1N1) (K173) and A/Memphis/8/88 (H3N2) (M88), they found that the 1918 HA was lethal by itself. They also found that the 1918 NA alone in the background of K173 and M88 was no more pathogenic than the parent strains. These studies suggested that the main lethal factor was the 1918 HA, not 1918 NA. We found that when 1918 HA and NA were placed together in the background of the low-pathogenic TX91 strain the virus was 100% lethal, whereas the parental TX91 strain was not. One of our objectives in this study was to determine the pathogenicity of recombinant viruses possessing genes from the 1918 pandemic influenza as compared to the highly-pathogenic avian influenza A/Vietnam/1203/2004 strain. The lung titers of mice challenged with the 1918 HA/NA:TX91 virus were comparable to A/Vietnam at all time points, with 1918 HA/NA:TX91 virus being higher at 24hours p.i. than the 1918 HA/NA/NS:TX91 virus.



The influenza NS1 protein is a known IFN-antagonist (40, 41, 25) and is required for influenza A virus virulence (25, 42). It has been shown that the binding of the NS1 viral protein to double-stranded RNA (dsRNA) (58) prevents the activation of multiple proteins, including PKR (43-45). PKR is activated by dsRNA (61) which in turn phosphorylates eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) (43). This prevents cellular translation from being turned off and viral proteins continue to be produced. PKR also plays a role in activating NF- $\kappa$ B (59) and IFN regulatory factor 3 (IRF-3) (41), both which are important transcription factors for the initiation of the IFN- $\alpha/\beta$  cascade. Another way NS1 prevents PKR activity is through directly binding the PKR protein (48). So, the binding of NS1 to PKR and dsRNA contributes to the pathogenicity of the virus by preventing the activation of the IFN- $\alpha/\beta$  cascade. This prevents the cells first line of defense against viral infection and allows the virus to overcome the initial immune response.

Another way the NS1 viral protein affects the host cell is by preventing the transport of mRNAs from the nucleus (63, 64). The NS1 protein binds to the poly(A) sequence at the 3' ends of mRNAs and inhibits the nuclear export of all poly(A)-containing viral and cellular mRNAs (65). NS1 not only prevents the export of mRNAs, but also prevents post-transcriptional splicing of pre-mRNAs (62). NS1 does this by binding to the U6 small nuclear RNA (snRNA) (62). NS1 allows the pre-mRNA to associate with the U1, U2, U4, U5, and U6 snRNAs to form spliceosomes but then remains associated with these spliceosomes to inhibit the catalytic steps of splicing that are normally carried out

by spliceosomal components (62). By preventing pre-mRNA splicing and mRNA transport the host cell cannot produce anti-viral proteins in the cytoplasm.

One of the distinctive clinical characteristics of the 1918 influenza virus was its ability to rapidly produce extensive damage to the respiratory epithelium (46). This is representative of a virus that is able to overcome the initial host immune response and to replicate to high titers and spread quickly from cell to cell. An NS1 protein that was especially effective at blocking the type I IFN system might have contributed to the exceptional virulence of the 1918 strain (25, 47).

We found that the addition of the 1918 NS, along with 1918 HA and NA, in the background of TX91 attenuated the virus. Lung titers in mice infected with 1918 HA/NA/NS1:TX91 were lower than 1918 HA/NA:TX91 or A/Vietnam at 24 hours, although the titers were still higher than mice challenged with TX91. Lung titers were comparable to TX91 lung titers at 72 hours. By 120 p.i., virus was undetectable in lungs of mice challenged with 1918 HA/NA/NS1:TX91 and TX91.

When the 1918 pandemic NS1 gene was placed in the background of the WSN strain of influenza, lung titers were higher than those challenged with the wild-type WSN virus (37). It has been shown that the NS1 protein of influenza is a virulence factor that interacts with the host immune system, attenuating the host immune response and thus allowing the virus to replicate unimpeded (25). We have demonstrated that NS1 originating from the 1918 pandemic virus attenuates viral replication in mice, as noted

previously (37). It has been mentioned that this could be due to the NS1 protein being host-specific (37).

The attenuating effect of NS1 in mice could be caused by relatively poor protein stability in mouse cells or by the inefficient interaction of either protein with specific host factors (37). The ability of NS1 to affect the host IFN response may involve the binding of RNA by NS1 (40, 41, 43) or its interaction with specific proteins, including PKR (48).

Possibly, the interactions of the 1918 NS1 protein with mouse PKR and/or other components of the type I IFN system is inefficient (48). The NS1 protein has been reported to interact with numerous other host-cell proteins (49-56). How the NS1 anti-IFN function is affected by interaction of NS1 with host-cell proteins is not clear (37).

The mouse immune system may also have a pathway not present in humans that allows it to circumvent the effects of the NS1 protein. The mouse may also lack some of the proteins that NS1 normally interacts with. This would limit the capability of the NS1 protein in its antagonism.

Viral titers for A/Vietnam remained high throughout the 120 hours examined suggesting that the immune system was attenuated, thus preventing the mice from clearing the virus by 120 hours post-infection.

The titers in lungs of mice challenged with TX91 were comparable to those challenged with the 1918 HA/NA/NS:TX91 virus suggesting that the HA and NA genes from the 1918 pandemic strain confer high pathogenicity to the virus.

## Summary

We have shown that the addition of the 1918 HA and NA genes to the low-pathogenic TX91 influenza strain increased its pathogenicity nearing that of the A/Vietnam H5N1 avian strain. Lung titers in mice challenged with 1918 HA/NA:TX91 were comparable to the highly pathogenic A/Vietnam strain at all time points of the study. All mice challenged with A/Vietnam died by day 7 whereas all mice challenged with 1918 HA/NA:TX91 died by day 11. As has been shown previously, when we added the 1918 NS to the TX91 the virus was attenuated. No mice died until day 8 in this group. It is worthwhile to further investigate the molecular interactions the NS1 viral protein has with host proteins in order to elucidate the cause of attenuation in mice, as well as virulence in humans. The NS1 protein could also be an attractive target for anti-viral treatments.

## References

1. Crosby, A. (1989) *America's Forgotten Pandemic* (Cambridge Univ. Press, Cambridge, U.K.).
2. Reid, A. H., Fanning, T. G., Hultin, J. V. & Taubenberger, J. K. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. (1999) *Proc. Natl. Acad. Sci. USA* 96:1651–1656
3. United States Department of Commerce (1976) *Historical Statistics in the United States: Colonial Times to 1970* (U.S. Govt. Printing Office), p. 58.
4. Dybing, J.K., Schultz-Cherry, S., Swayne, D.E., Suarez, D.L., Perdue, M.L. Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J Virol.* 2000 Feb;74(3):1443-50.
5. Taubenberger, J.K. Influenza virus hemagglutinin cleavage into HA1, HA2: no laughing matter. *Proc Natl Acad Sci U S A.* 1998 Aug 18;95(17):9713-5
6. Ogawa, T. & Ueda, M. Genes involved in the virulence of an avian influenza virus. *Virology* 113, 304-313 (1981)
7. Bosch, F.X., Orlich, M., Klenk, H.K., Rott, R. The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. *Virology* 95, 197-207 (1979)
8. Hatta, M., Gao, P., Halfmann, P. & Kawaoka, Y. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293, 1840-1842 (2001)

9. Taubenberger, J.K., Reid, A.H., Krafft, A.E., Bijwaard, K.E. & Fanning, T.G. Initial genetic characterization of the 1918 “Spanish” influenza virus. *Science* 275, 1793-1796 (1997)
10. Reid A.H., Fanning, T.G., Janczewski, T.A. and Taubenberger, J.K. (2000). Characterization of the 1918 “Spanish” influenza virus neuraminidase gene. *Proc. Natl. Acad. Sci. USA* 97:6785-6790
11. Glaser, L., Stevens, J., Zamarin, D., Wilson, I.A., García-Sastre, A., Tumpey, T.M., Basler, C.F., Taubenberger, J.K., Palese, P. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *J Virol.* 2005 Sep;79(17):11533-6.
12. Tumpey, T.M., Maines, T.R., Van Hoeven, N., Glaser, L., Solórzano, A., Pappas, C., Cox, N.J., Swayne, D.E., Palese, P., Katz, J.M., García-Sastre, A. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science.* 2007 Feb 2;315(5812):655-9.
13. Tumpey, T.M., Basler, C.F., Aguilar, P.V., Zeng, H., Solórzano, A., Swayne, D.E., Cox, N.J., Katz, J.M., Taubenberger, J.K., Palese, P., García-Sastre, A. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science.* 2005 Oct 7;310(5745):77-80.
14. Kash, J.C., Tumpey, T.M., Proll, S.C., Carter, V., Perwitasari, O., Thomas, M.J., Basler, C.F., Palese, P., Taubenberger, J.K., García-Sastre, A., Swayne, D.E., Katze, M.G. Genomic analysis of increased host immune and cell death responses

induced by 1918 influenza virus. *Nature*. 2006 Oct 5;443(7111):578-81. Epub 2006 Sep 27.

15. Kobasa, D., Takada, A., Shinya, K., Hatta, M., Halfmann, P., Theriault, S., Suzuki, H., Nishimura, H., Mitamura, K., Sugaya, N., Usui, T., Murata, T., Maeda, Y., Watanabe, S., Suresh, M., Suzuki, T., Suzuki, Y., Feldmann, H., Kawaoka, Y. Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. *Nature*. 2004 Oct 7;431(7009):703-7.
16. Francis, T. Jr., Moore A.E., A study of the neurotropic tendency in strains of the virus of epidemic influenza. *J. Exp. Med.* 1940 72: 717-728
17. Castrucci, M.R., Kawaoka, Y. Biologic importance of neuraminidase stalk length in influenza A virus. *J Virol*. 1993 Feb;67(2):759-64.
18. A.H. Reid, T.A. Janczewski, R.M. Lourens, A.J. Elliot, R.S. Daniels, C.L. Berry, J.S. Oxford and J.K. Taubenberger. (2003) *Emerging Infectious Disease*. Vol. 9, No. 10
19. Palese, P., Compans, R.W. Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-triinfluenzaoroacetylneuraminic acid (FANA): mechanism of action. *J Gen Virol*. 1976 Oct;33(1):159-63.
20. Burnet, F.M., Stone, J.D. The receptor-destroying enzyme of *Vibrio cholera*. (1948) *Aust. J. Exp. Biol. Med. Sci.* 25, 227–233.
21. Colman, P.M., Varghese, J.N., Laver, W.G. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature*. 1983 May 5-11;303(5912):35-40.

22. Webster, R.G., Hinshaw, V.S., Laver, W.G. Selection and analysis of antigenic variants of the neuraminidase of N2 influenza viruses with monoclonal antibodies. *Virology*. 1982 Feb;117(1):93-104.
23. Jahiel, R.I., Kilbourne, E.D. Reduction in plaque size and reduction in plaque number as differing indices of influenza virus-antibody reactions. *J Bacteriol*. 1966 Nov;92(5):1521-34.
24. Kilbourne, E. Influenza pandemics in perspective. *JAMA*. 1977 Mar 21;237(12):1225-8.
25. García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D.E., Durbin, J.E., Palese, P., Muster, T. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology*. 1998 Dec 20;252(2):324-30.
26. Seo S.H., Hoffmann, E., Webster, R.G. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nat Med*. 2002 Sep;8(9):950-4. Epub 2002 Aug 26.
27. Pleschka, S., Jaskunas, R., Engelhardt, O.G., Zürcher, T., Palese, P., García-Sastre, A. A plasmid-based reverse genetics system for influenza A virus. *J Virol*. 1996 Jun;70(6):4188-92.
28. Radecke, F., Spielhofer, P., Schneider, H., Kaelin, K., Huber, M., Dötsch, C., Christiansen, G., Billeter, M.A. Rescue of measles viruses from cloned DNA. *EMBO J*. 1995 Dec 1;14(23):5773-84.
29. Garcia-Sastre, A., Palese, P. Genetic Manipulation of Negative-Strand RNA Virus Genomes. *Annu. Rev. Microbiol*. 1993. 47:765-90



30. Enami, M., Luytjes, W., Krystal, M. & Palese, P. Introduction of site-specific mutations into the genome of influenza virus. *Proc Natl Acad Sci U S A*. 1990 May;87(10):3802-5.
31. Neumann, G., Zobel, A., Hobom, G. RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* 202, 477-479 (1994)
32. Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A*. 1999 Aug 3;96(16):9345-50.
33. Schulman, J.L., and Palese, P. 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. *J Virol*. 1977 Oct;24(1):170-6.
34. Li S, Schulman J, Itamura S, Palese P. Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. *J Virol*. 1993 Nov;67(11):6667-73.
35. Kobasa, D., Jones, S.M., Shinya, K., Kash, J.C., Copps, J., Ebihara, H., Hatta, Y., Kim, J.H., Halfmann, P., Hatta, M., Feldmann, F., Alimonti, J.B., Fernando, L., Li, Y., Katze, M.G., Feldmann, H., Kawaoka, Y. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature*. 2007 Jan 18;445(7125):319-23.
36. Fodor, E., Devenish, L., Engelhardt, O.G., Palese, P., Brownlee, G.G., Garcia-Sastre, A. Rescue of influenza A virus from recombinant DNA. *J Virol*. 1999 Nov;73(11):9679-82.

37. Basler, C.F., Reid, A.H., Dybing, J.K., Janczewski, T.A., Fanning, T.G., Zheng, H., Salvatore, M., Perdue, M.L., Swayne, D.E., García-Sastre, A., Palese, P., Taubenberger, J.K. Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc Natl Acad Sci U S A*. 2001 Feb 27;98(5):2746-51.
38. Lu, X., Tumpey, T.M., Morken, T., Zaki, S.R., Cox, N.J., Katz, J.M. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J Virol*. 1999 Jul;73(7):5903-11.
39. Tumpey, T.M., Lu, X., Morken, T., Zaki, S.R., Katz, J.M. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. *J Virol*. 2000 Jul;74(13):6105-16.
40. Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A.A., García-Sastre, A. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol*. 2000 Dec;74(24):11566-73.
41. Talon, J., Horvath, C.M., Polley, R., Basler, C.F., Muster, T., Palese, P., García-Sastre, A. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol*. 2000 Sep;74(17):7989-96.
42. Talon, J., Salvatore, M., O'Neill, R.E., Nakaya, Y., Zheng, H., Muster, T., García-Sastre, A., Palese, P. Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. *Proc Natl Acad Sci U S A*. 2000 Apr 11;97(8):4309-14.

43. Lu, Y., Wambach, M., Katze, M.G., Krug, R.M. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology*. 1995 Dec ;214(1):222-8.
44. Hatada, E., Saito, S., Fukuda, R. Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells. *J Virol*. 1999 Mar;73(3):2425-33.
45. Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P., Muster, T. influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol*. 2000 Jul;74(13):6203-6.
46. Winternitz, M.C., Wason, I.M., McNamara F.P. Pathology of influenza. (1920) Yale University Press, New Haven, CT.
47. García-Sastre, A., Durbin, R.K., Zheng, H., Palese, P., Gertner, R., Levy, D.E., Durbin, J.E. The role of interferon in influenza virus tissue tropism. *J Virol*. 1998 Nov;72(11):8550-8.
48. Tan, S.L., Katze, M.G. Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. *J Interferon Cytokine Res*. 1998 Sep;18(9):757-66.
49. Chen, Z., Li, Y., Krug, R.M. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. *EMBO J*. 1999 Apr 15;18(8):2273-83.

50. Nemeroff, M.E., Barabino, S.M., Li, Y., Keller, W., Krug, R.M. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3'end formation of cellular pre-mRNAs. *Mol Cell*. 1998 Jun;1(7):991-1000.
51. Qiu, Y., Krug, R.M. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol*. 1994 Apr;68(4):2425-32.
52. Qiu, Y., Nemeroff, M., Krug, R.M. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. *RNA*. 1995 May;1(3):304-16.
53. Aragón, T., de la Luna, S., Novoa, I., Carrasco, L., Ortín, J., Nieto, A. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. *Mol Cell Biol*. 2000 Sep;20(17):6259-68.
54. Marión, R.M., Zürcher, T., de la Luna, S., Ortín, J. Influenza virus NS1 protein interacts with viral transcription-replication complexes in vivo. *J Gen Virol*. 1997 Oct;78 ( Pt 10):2447-51.
55. Falcón, A.M., Fortes, P., Marión, R.M., Beloso, A., Ortín, J. Interaction of influenza virus NS1 protein and the human homologue of Staufen in vivo and in vitro. *Nucleic Acids Res*. 1999 Jun 1;27(11):2241-7.
56. Wolff, T., O'Neill, R.E., Palese, P. Interaction cloning of NS1-I, a human protein that binds to the nonstructural NS1 proteins of Influenza A and B viruses. *J Virol*. 1996 Aug;70(8):5363-72.

57. Tumpey, T.M., García-Sastre, A., Mikulasova, A., Taubenberger, J.K., Swayne, D.E., Palese, P., Basler, C.F. Existing antivirals are effective against Influenza viruses with genes from the 1918 pandemic virus. *Proc Natl Acad Sci U S A*. 2002 Oct 15;99(21):13849-54.
58. Hatada E and Fukuda R. Binding of Influenza A virus NS1 protein to dsRNA *in vitro*. *J. Gen. Virol.* 1992 73. 3325-3329
59. Zamanian-Daryoush, M., T. H. Mogensen, J. A. DiDonato, and B. R. Williams. NF- $\kappa$ B activation by double-stranded-RNA-activated protein kinase (PKR) is mediated through NF- $\kappa$ B-inducing kinase and I $\kappa$ B kinase. *Mol. Cell. Biol.* 2000 20:1278-1290.
60. Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, García-Sastre A. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. *J Virol.* 2000 Dec;74(24):11566-73.
61. Williams, B. R. PKR: a sentinel kinase for cellular stress. *Oncogene* 1999 18:6112–6120.
62. Y Lu, X Y Qian and R M Krug. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes Dev.* 1994 8: 1817-1828
63. Nucleocytoplasmic transport: the influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. Alonso-Caplen FV, Nemeroff ME, Qiu Y, Krug RM. *Genes Dev.* 1992 Feb;6(2):255-67.

64. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. Fortes P, Beloso A, Ortín J. EMBO J. 1994 Feb 1;13(3):704-12.
65. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). Qiu Y, Krug RM. J Virol. 1994 Apr;68(4):2425-32.