Temperature Sensitivity of Avian, Swine, and Human Influenza A Viruses and Induction of Innate Immunity in a Novel Porcine *in vitro* Model.

by

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A Thesis submitted to the Faculty of Graduate Studies of

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Temperature Sensitivity of Avian, Swine, and Human Influenza A Viruses and Induction of Innate Immunity in a Novel Porcine *in vitro* Model

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Abstract

Pigs are considered important in interspecies transmission of influenza A viruses as their respiratory tracts contain receptors used by both avian and mammalian-tropic viruses. Residue 627 of the viral polymerase basic protein 2 (PB2) is thought to determine temperature sensitivity in certain mammalian systems, but its role has yet to be investigated in a swine system. Ten influenza viruses from avian, human and swine hosts were evaluated for their ability to grow at 33°C, 37°C and 41°C in an immortalized swine alveolar macrophage cell line. Of TNF-α, IL-1β, and IFN-α, only TNF-α was induced following 48 hours of infection, in response to only certain viruses. Temperature-dependent growth of each virus was not consistently correlated to species of isolation, and PB2 residue 627 did not dictate temperature preference. These results suggest that, in a swine system, factors allowing for efficient replication at various temperatures are likely polygenic, possibly of viral and host origin.

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List of Abbreviations Used

293T – Human embryonic kidney cell line

AF – Allantoic fluid

AMEM – Alpha Modified Eagle's Medium

ANOVA – Analysis of variance

ATCC – American Type Culture Collection

BSA – Bovine Serum Albumin

CD – Cluster of differentiation marker

CFIA – Canadian Food Inspection Agency

CL – Containment Level

CMC – Carboxymethylcellulose

CO_2 - Carbon dioxide

CPE – Cytopathic effect

cRNA – Positive-sense copy of viral RNA

CTL – Cytotoxic T-lymphocyte

DC – Dendritic cell

DMEM- Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

dsRNA – Double stranded RNA

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal Bovine Serum

FITC – Fluorescein isothiocyanate

HA0 – Uncleaved hemagglutinin precursor

HA1 – Cleaved hemagglutinin subunit 1

HA2 – Cleaved hemagglutinin subunit 2

HA – Hemagglutinin

HAs – Hemagglutination Assays

HAU – Hemagglutination units

IFN – Interferon

Ig – Immunoglubulin

IL – Interleukin

IPAM – Immortalized porcine alveolar macrophage

IRF – Interferon regulatory factor

LB – Luria Bertani

M – Matrix gene

MAA - Maackia Amurensis Lectin II

MDCK – Mandin-Darby Canine Kidney

MHC – Major histocompatibility complex ml – Milliliter

μl – microlitre

ml – millilitre

mRNA – Messenger ribonucleic acid

NA – Neuraminidase

NCFAD – National Centre for Foreign Animal Disease

NLS – Nuclear localization signal

NP – Nucleoprotein

NS – Non-structural protein

PA – Polymerase acidic protein

PB1 – Polymerase Basic Protein 1

PB2 – Polymerase Basic Protein 2

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PE – Phycoerythrin

PFU – Plaque forming unit

pPol I – Polymerase I-driven plasmids

RNA – Ribonucleic acid

RNP – Ribonucleoprotein

RPM – Revolutions per minute

RT-PCR – Reverse transcriptase polymerase chain reaction

SA – Sialic acid

SIV – Swine influenza virus

SNA - Sambuccus nigra lectin

SWC – Swine workshop cluster number

$TCID_{50}$ – 50% Tissue culture infectious dose

TNF – Tumor necrosis factor

TPCK - L-1-tosylamido-2-phenylethyl chloromethyl ketone

vRNA – Viral ribonucleic acid

w/v – Weight/volume

Introduction

Influenza Virus

1. Classification and Structure

Influenza A is one of the five genera found in the *Orthomyxoviridae* family, which also includes influenza B, influenza C, *Isavirus* and *Thogotovirus*, characterized as enveloped viruses with a negative sense, single-stranded, segmented RNA genome. The name *Orthomyxoviridae* comes from the Greek words *orthos* and *myxa,* meaning "standard" and "mucus", respectively, and accordingly influenza viruses cause disease of the mucosal systems. The nomenclature system for these viruses follows a standard format, starting with the specific genus and followed by species of isolation, geographical location, isolate number, and year of isolation, followed by the virus subtype in parentheses. An example of this is A/Chicken/Vietnam/14/2005 (H5N1), indicating the virus is type A influenza of chicken origin from Vietnam, given the isolate number of 14, isolated in 2005 and is of the H5 hemagglutinin type and N1 neuraminidase type. The naming system for most human influenza viruses follows the same format but does not include the species of isolation (Palese & Shaw, 2006; Wright *et al.,* 2006).

A total of eight gene segments encoding up to eleven polypeptides make up the influenza A virus genome, and each segment is packaged in a nucleocapsid with an associated polymerase complex. Segments are numbered 1 through 8, going from largest to smallest. Five of the eight genes encode for a single protein, while the other three encode for an additional polypeptide. The PB1 gene of some influenza isolates encodes a second protein using an alternate reading frame, while segments 7 and 8 of all influenza A viruses encode for two proteins using alternative splicing of viral mRNA (Cheung &

Poon 2007). A full list of viral segments, their associated gene products, and putative major functions is found in Table 1.

Virions are pleiomorphic, as both spherical and rod-shaped particles measuring 100-300 nm in diameter have been observed. The influenza A virus has a complex structure of cellular and viral components. The surface of the virus particle is heavily covered by two viral glycoproteins, the hemagglutinin (HA) and neuraminidase (NA), which protrude like spikes from the viral envelope at a ratio of approximately 400 HA: 100 NA molecules. A third viral protein, M2, is embedded in the envelope and creates a channel between the virus interior and external milieu. In addition to its three viral protein components, the outer membrane is a lipid bilayer made of host cell-derived lipids obtained during budding. Immediately below the envelope lies the M1 layer, composed of the viral matrix protein (M1), which interacts with cytoplasmic tails of the envelope glycoproteins as well as the NS2 protein and ribonucleoprotein (RNP) complexes on the interior. All eight vRNA segments are packaged as individual RNP complexes, which consist of genomic RNA encapsidated by nucleoprotein and associated with all three proteins of the polymerase complex. These segments are also specifically packaged in the virion, with seven segments forming and organized ring structure and one segment found in the center (Cheung & Poon, 2007; Noda *et al.,* 2006; Palese & Shaw, 2006).

Table 1. Summary of influenza A virus gene segments, their relative sizes, encoded proteins and associated functions.

Gene Segment	Relative Size [a] (bps)	Gene Products	Major Putative Function [b]
PB2	2341	PB2 Polymerase	Cap-snatching of host pre-mRNAs for viral transcription
PB1	2341	PB1 Polymerase	RNA-dependent RNA Polymerase, Transcription and Translation of genome
		PB1-F2 protein	Apoptosis of host alveolar macrophages
PA	2233	PA Polymerase	Endonuclease activity
HA	1778	Hemagglutinin envelope glycoprotein	Receptor-mediated recognition of host cells, fusion of viral and endosomal membranes
NP	1565	Nucleoprotein	Viral RNA encapsidation, protection of nascent RNA strands during replication, nuclear import of incoming vRNPS
NA	1413	Neuraminidase envelope glycoprotein	Sialidase activity – cleaves sialic acid from HA and NA molecules during budding process, prevents virion aggregation
M	1027	M1 – Matrix Protein	Inhibits viral mRNA transcription, Role in nuclear export of RNPs, Primary mediator of virus assembly, determines virion morphology
		M2 – Ion Channel Protein	Allows proton influx from acidified endosomes, allows for pH-mediated fusion of viral and endosomal membranes
NS	890	NS1 – Non-structural Protein	Suppressor of innate immunity; prevents dendritic cell maturation and type I interferon response, Inhibits host cell transcription
		NS2 – Nuclear export protein	Nuclear export of nascent RNPs from infected cells

[a] Sizes based on sequence of A/PR/8/34 (H1N1) vRNA (Cheung & Poon, 2007).
[b] Based on information from Wright *et al.,* 2006; Cheung & Poon, 2007; Dias *et al.,* 2009.

2. Life Cycle

2.1. Entry and Uncoating

The virus life cycle consists of several phases, the first of which involves entry into the host cell and liberation of the viral genetic material. This process begins with receptor recognition and binding by the viral hemagglutinin (HA). Attachment is mediated by an interaction between the receptor binding site of the HA molecule and sialic acid (SA)-containing glycoconjugates, the receptor molecules, on target cells. The precise nature of the SA linkage is the major determinant for recognition by the HA; where avian-tropic viruses preferentially recognize and bind to SAs with an alpha-2,3 linkage to the carbohydrate while human-tropic viruses bind SAs with alpha-2,6 linkages. The specificity of these reactions goes even further beyond the linkage type, as the degree of sulfation, fucosylation, and sialylation on the terminal sugar molecules further influence binding preference of HAs from different viruses (Stevens *et al.,* 2006).

Following receptor binding, the virus is internalized into endosomes, either through clathrin-coated pits or a clathrin and caveolin-independent pathway (Maines *et al.,* 2008). The uncoating process requires a low pH-induced fusion between the viral envelope and endosomal membrane. This is initiated by M2, an integral membrane protein on the viral envelope in a homotetramer configuration, which act as an ion channel. Protons enter the virion from the endosome through the M2 channel, leading to virion acidification and destabilization of the M1 layer. This also induces a conformational change in the HA, leading to fusion of the viral and endosomal membranes (Palese & Shaw, 2006).

2.1.1. Hemagglutinin

The HA glycoprotein exists in a homotrimer configuration and covers the surface of the influenza virion. HA serves two main functions in the virus life cycle: attachment to SA-containing host cell receptors and fusion with host cell endosomal membranes following internalization, leading to virus uncoating. HA is produced as an uncleaved precursor molecule called HA0, which requires post-translational cleavage into a functionally active molecule consisting of HA1 and HA2 subunits (Palese & Shaw, 2006). Cleavage of HA0 is essential for the virus life cycle, as uncleaved HAs lead to non-productive infection since fusion with endosomal membranes is inhibited (Cheung & Poon, 2007; Nayak *et al.,* 2004). The M2-mediated acidification of the virion causes a conformational change in the HA molecule, exposing a 19 amino acid-long fusion peptide that anchors with the endosomal membrane. The combined fusion of several HA molecules with the endosomal membrane creates a pore through which the RNP complexes are released into the cytoplasm, completing the uncoating process (Palese Shaw, 2006).

2.2. Replication

After release into the host cell, viral RNP complexes migrate to the nucleus via nuclear localization signals (NLS) found on the nucleoprotein (NP), though NLS have also been found on the three associated polymerase proteins, which likely contribute to migration as well. Nuclear entry is an energy-dependent process and involves the interaction of NP with alpha importin proteins, which then deliver the RNP through the

nuclear pore. Unlike most RNA viruses, influenza viruses are unique in that they undergo transcription and replication within the nucleus.

2.2.1. Transcription

The first step in the generation of viral progeny is transcription, a primer-dependent process. The current model holds that transcription is initiated by the binding of PB1 from the incoming RNP to the 5' terminal end of the vRNA, inducing conformational changes in both PB1 and PB2. This activates PB2's cap-snatching activity, causing it to seek out and bind to host cell pre-mRNA (m^7GpppX^m-containing) transcripts that are used as primers for viral mRNA synthesis. PB1 then binds to the 3' terminal end of the vRNA molecule and cleaves of the PB2-bound host pre-mRNA molecule approximately 10-13 nucleotides from its 5' end. A guanine residue is then added to the capped primer as the initiation step for transcription. Elongation of the newly synthesized mRNA continues as the vRNA is threaded through the polymerase complex in a 3' to 5' direction, but the 5' end remains bound to the PB1 subunit. This causes the polymerase to stutter on a stretch of 5-7 uridines near the 5' end of the vRNA, leading to polyadenylation at the 3' end of the nascent mRNA transcript and subsequently terminating the transcription process (Cheung & Poon, 2007; Palese & Shaw, 2006). In recent months, two separate groups have suggested that the endonuclease activity in fact lies in the amino terminal of PA rather than on PB1, and therefore the current model of transcription requires updating (Dias *et al.,* 2009; Yuan *et al.,* 2009).

2.2.2. Protein Synthesis

Synthesis of the HA and NA, and M2 glycoproteins occurs on membrane-bound ribosomes, from where they enter the endoplasmic reticulum and are transported through the trans-Golgi network. The remaining viral proteins undergo synthesis in the cytoplasm, from which point they migrate back into the nucleus via nuclear localization signals to carry out their various functions (Palese & Shaw, 2006). Once vRNA replication has commenced, transcripts of the HA, NA and M genes are the predominant species, while the three polymerase proteins are generally under-represented at all time-points. The M1 polypeptide has an inhibitory role on transcription and is also involved in nuclear export of RNPs, and therefore its expression must be delayed until later times post-infection. The increase in transcription of structural genes at later times also indicates that RNA synthesis has reached a critical level and that morphogenesis and assembly can begin (Palese & Shaw, 2006).

2.2.3. Regulation of Transcription and Genome Replication

It was long believed that a switch exists in the virus life cycle, changing from a transcription state to a replication state. More recently, a new model of transcription and replication was proposed that suggests that there is no definitive switch, but rather that until a sufficient amount of nascent polymerase and NP are generated in the infected cell to encapsidate the vRNA, they are degraded and only mRNA transcription takes place. Therefore, early time-points post-infection are predominated by mRNA synthesis, but later on the balance shifts towards genome replication (Palese & Shaw, 2006; Nayak *et al.,* 2004, Cheung & Poon, 2007).

2.2.4. vRNA Replication and Nuclear Export of RNPs

Genome replication is a two-step, primer independent process and results in a full-length duplication of the vRNA. First, a full-length positive sense copy of the segment is generated (cRNA), which is then used to transcribe a negative sense copy identical to the original strand (vRNA). Nascent NP proteins generated in the infected cells migrate back into the nucleus and encapsidate the cRNA and vRNA, but not mRNA, strands as they are synthesized by the polymerase. This interaction is thought to stabilize the strands of RNA and is especially important for allowing the polymerase to read through the entire segment and preventing the formation of a poly-A tail. The interaction of the vRNA segment with PB1 domains likely differs in the pre-transcription and pre-replication states, dictating which of the processes will take place (Palese & Shaw, 2006; Cheung & Poon, 2007).

Following sufficient vRNA replication and nuclear assembly of the RNP complexes these structures are exported from the nucleus for packaging into newly forming virions. RNP export relies on interactions with several viral gene products and host factors, especially those that interact with nucleoporins (Nayak *et al.*, 2004). Following its synthesis late in the virus replication cycle, M1 enters the nucleus where it associates with RNP complexes. A second viral protein, NS2, also enters the nucleus and binds the RNP-associated M1 proteins. NS2, also termed the nuclear export protein, contains a nuclear export signal and interacts with cellular nuclear export factor Crm1 as well as many nucleoporins. The current model therefore suggests a vRNP-M1-NS2 protein complex is required for the nuclear export of newly synthesized vRNPs (Nayak *et al.,* 2004; Palese & Shaw, 2006).

2.3. Assembly, Budding and Release

The majority of viral proteins, including the three polymerase proteins, NS1, NS2, NP and M1, are synthesized on ribosomes in the cytoplasm of infected cells, from which point they enter the nucleus to carry out their designated functions. Unlike these, the three envelope proteins (HA, NA, and M2) are synthesized on membrane-associated ribosomes and are transported through the trans-Golgi network where they undergo post-translational processing and are delivered to the budding site. For viruses whose HA's contain a polybasic HA0 cleavage site, the trans-Golgi network also serves to cleave the HA precursors into HA1 and HA2 due to the presence of furin. HA and NA contain strong apical sorting signals and are delivered to the apical surfaces of polarized cells. Specifically, these two proteins have a high affinity for lipid rafts and selectively accumulate at these sites. The M2 protein is found not within the rafts but between raft domains, likely acting as a linker molecule (Nayak *et al.,* 2004; Palese & Shaw, 2006).

The precise mechanism by which the vRNPs and remaining viral proteins traffic to the assembly site remains unclear, though M1 is thought to play a role in directing the vRNPs, and M2 may be involved in capturing the vRNPs once they reach the apical area (Palese & Shaw, 2006). Since the genome is segmented, all eight segments must be packaged into progeny virions for them to be infectious. Influenza viruses contain highly conserved non-coding regions at their 3' and 5' termini, and these are thought to direct packaging (Muramoto et al, 2006). Bud formation is regulated by the accumulation of M1 protein beneath the apical membrane, and M1 alone is capable of driving virus-like particle formation. When the virion pinches off from the host cell membrane, it is not yet free to infect other cells. HA trimers on the newly budded virus become attached to the

same receptor molecules used for cell entry, causing the viruses to stick to their host cells upon budding (Nayak *et al.,* 2004; Palese & Shaw, 2006). Efficient release relies on the action of another envelope protein, the neuraminidase (NA).

2.3.1. Neuraminidase

Neuraminidase (NA) is the second most abundant viral envelope protein and exists in a homotetramer configuration. Like HA, NA also has an affinity for host SA - containing glycolipids and glycoproteins but it functions to enzymatically cleave these molecules. The sialidase activity of NA allows efficient virus release from the host cell after budding, preventing virion aggregation on the host cell surface. This property also implicates NA in allowing the virus to pass through the respiratory tract and reach target cells, as mucins in the tract are laden with SA-containing species, which could sequester incoming viruses if the HA molecules bound to them with no means of release (Palese & Shaw, 2006; Nayak *et al.*, 2004). Another putative function of NA is assisting in the activation of HA0 into HA1 and HA2. The NA of A/WSN/33 was shown to bind the protease precursor plasminogen, which led to higher concentrations of plasmin in the area around the virus and subsequently higher levels of enzymatic activation (Goto & Kawaoka, 1998). This property allows for increased activation of HAs that contain only a single basic residue, enhancing the pathogenicity of such viruses in mice (Goto *et al.,* 2001).

3. Influenza in Humans

3.1. Disease – Organ and Cell Tropism

Influenza viruses are transmitted primarily via aerosol droplets of approximately 2 microns in size or smaller, which are generated by coughing and sneezing (Wright *et al.*, 2006). In humans, the virus replicates exclusively in the respiratory tract where α-2,6-linked sialic acid receptors are found on epithelial cells of the nasal mucosa, paranasal sinuses, pharynx, trachea and bronchi. Deep within the lung the virus can also infect ciliated alveolar epithelial cells in addition to alveolar macrophages and dendritic cells (Ibricevic *et al.*, 2006; Shinya *et al.*, 2006). Following a typical three day incubation period a range of clinical signs become apparent, with common symptoms including sneezing, cough, nasal congestion, fever, malaise, sore throat, chills, anorexia, myalgia and headache. Illness severity is correlated with levels of virus shedding, both peaking at approximately 48 hours and declining until the seventh day, at which time little shed virus is detectable. However, virus shedding can last nearly twice as long in children and typically reaches higher titers than in adults (Nguyen-Van-Tram, 1998; Wright *et al.*, 2006).

3.2. Immune Response

The primary site of human influenza virus infection is the respiratory tract, which is at constant risk of exposure to respiratory pathogens through the act of inhalation. For this reason, the lungs are equipped with several lines of defense to prevent infections from occurring. The mucus within the respiratory tract acts as a physical barrier to infection as it is laden with molecules similar in structure to the SA-containing receptors

used by the virus. When HA glycoproteins of incoming virions bind to these receptor analogs they are unable to interact with receptor molecules on host cells and thus entry is prevented (Tamura *et al.,* 2004). Circulating protease inhibitors and the act of ciliary beating also likely contribute to preventing infection (Wright *et al.,* 2006). In addition to physical barriers, the respiratory tract contains key elements of the innate immune system to recognize and eliminate airborne pathogens like influenza virus. These include pattern-recognition receptors that respond to different pathogen-associated molecular patterns, cytokines and chemokines, and leukocytes such as macrophages, dendritic cells, and natural killer cells.

Upon infection of pulmonary epithelial cells, viral dsRNA is recognized by Toll-like receptor 3 (TLR-3), which stimulates the production of Type 1 Interferons (IFN) (Wright *et al.,* 2006). Interferons are found at high levels in nasal and pulmonary secretions following infection with influenza and are correlated with peak viral titers and symptom scores in experimentally infected humans (Hayden *et al.,* 1998; Tamura & Kurata, 2004). IFN-α and IFN-β play an integral role in inhibiting virus spread, acting as warning signals to nearby uninfected cells to produce enzymes that block viral replication. Additionally, they affect the adaptive immune response; enhancing cytotoxic T lymphocyte (CTL)-mediated killing by promoting class I major histocompatibility complex (MHC) expression on the surface of infected cells (Abbas & Lichtman, 2003).

3.2.1. Alveolar Macrophages

Alveolar macrophages are key players in connecting the innate and adaptive immune response to influenza virus infection. As residents of the air spaces in alveoli,

alveolar macrophages are among the first cells to encounter influenza virus and virus-infected epithelial cells, and, therefore, play a crucial role in the initial response to these infections (Tumpey *et al.,* 2005). In addition to phagocytosing cells killed by virus infection, i*n vivo* and *in vitro* data has shown these cells can also be directly infected with influenza virus and secrete a number of cytokines in response (Chan *et al.,* 2005). Tumor Necrosis Factor-α (TNF-α) is a key cytokine of the innate immune response produced primarily by activated macrophages in response to influenza virus infections (Seo & Webster, 2002). Its main biologic actions are to recruit monocytes and neutrophils to the site of infection by inducing vascular endothelial cells to express leukocyte adhesion molecules (Abbas & Lichtman, 2003). Alveolar macrophages are derived from monocytes, which circulate in the blood and differentiate to tissue-specific macrophages after reaching the lungs (Fels *et al.,* 1986). At different stages in their differentiation, these cells express different surface molecules, including cluster or differentiation (CD) receptors and chemokine receptors, causing them to vary in their abilities to respond to certain antigens (Gordon & Taylor, 2005). Using a swine monocyte-macrophage model it was shown that infection with African swine fever virus increased in cells with up-regulated expression of mature macrophage markers, indicating that stages of differentiation are not only important in effector capabilities but also influence susceptibility to viral infection (McCullough *et al.*, 1999).

3.2.2. Adaptive Immune Response

The short duration of influenza infection presents the importance of a successful innate immune response. However, complete recovery from infection relies on the cells

of the adaptive immune system that specifically target the infecting virus. An effective adaptive response involves the individual and coordinated actions of B and T lymphocytes. Anti-influenza antibodies produced by B cells play a critical role in protection from re-infection, while cellular-mediated responses are largely responsible for clearing an active infection by targeted killing of infected cells (Wright *et al.*,2006).

Influenza virus infection results in the production of antibodies against HA, NA, NP and M viral proteins, though traditionally only anti-HA and anti-NA antibodies were thought to confer protection from further infections (Wright *et al.*, 2006). However, recent studies on heterosubtypic immunity have suggested that antibodies generated against the M2 glycoprotein are protective, though these results are based on vaccine work with M2 conjugated to an adjuvant, and whether such protective antibodies can be generated during a natural infection is unclear (Wu *et al.*, 2008). IgG and IgA antibodies generated against one of the five antigenic regions on the HA1 subunit neutralize the virus to prevent infection, whereas those targetting NA prevent virus release from infected cells (Thomas *et al.*, 1998; Wright *et al.*, 2006). The antibody-mediated adaptive response is highly protective against infection by viruses with antigenically similar glycoproteins to those against which the antibodies were generated, and the annual influenza vaccination program aims to generate such a response. Antibody-mediated protection can last for many years, and a recent study of 1918 pandemic survivors found functional, circulating memory B cells that produced neutralizing antibodies against the virus *in vitro* (Yu *et al.*, 2008). However, as seen in the case of vaccination-induced antibodies, the continual accumulation of antigenic changes on the HA of circulating viruses from year to year allows for infection in previously vaccinated individuals,

indicating antibody-mediated protection is incomplete (Wright *et al.,* 2006, Thomas *et al.,* 1998).

Cell-mediated immunity is also crucial in recovery from influenza virus infection and is mediated by a CD8+, Type 1-helper T cell (T_H1) dominated response (Fernandez-Sesma *et al.,* 2006). Virus-specific CTLs begin proliferating in the regional lymph nodes by 3-4 days post-infection and migrate to the lungs shortly thereafter (La Gruta & Doherty, 2006). The mechanism by which they mediate killing of infected cells is not entirely clear, and may involve both direct cytolytic killing and the production and secretion of antiviral cytokines. Additionally, since T cells react to processed viral peptides rather than antigenic surface structures, they respond to a variety of viral epitopes including those from surface glycoproteins and internal proteins. This allows for cross-reactive protection from viruses of different subtypes when CTLs are targeted against peptides of internal proteins in addition to protection from homologous viruses if cells are specific for certain HA or NA epitopes. CD4+ T cells are also involved in the immune response, with T_H1 cells secreting IFN-γ to aid in CTL-mediated killing, and T_H2-mediated responses directing B cells to produce antibodies against the active infection (Fernandez-Sesma *et al.,* 2006; Wright *et al.,* 2006).

3.2.3. Dysregulated Immune Responses

The purpose of the innate immune response is to create an environment inhospitable to virus replication and to recruit specific immune cells and factors to neutralize free virus particles and kill virus-infected cells. Many of the cytokine effector molecules secreted by cells of the innate immune system are pro-inflammatory and cause a degree of damage to the host. A number of influenza isolates are associated with

inducing an exaggerated immune response, leading to severe disease and high-level mortality. Fatal human cases of H5N1 influenza have been associated with hypercytokinemia, which has been implicated in causing severe disease. Experimental H5N1 infections of human primary monocyte-derived macrophages *in vitro* resulted in significantly enhanced TNF-α and IFN-β production compared to infection with human H3N2 or H1N1 viruses (Cheung *et al.,* 2002; Maines *et al.,* 2008), and such responses were also seen in H5N1-infected mice (Szretter *et al.,* 2007). Similar to H5N1 viruses, experimental infection of mice and non-human primates with the rescued 1918 pandemic influenza virus resulted in an exaggerated cytokine response, which was associated with disease severity and death (Kobasa *et al.,* 2007; Perrone *et al.,* 2008).

3.2.4. Viral Suppressors of Immunity - NS1 and PB1-F2

While the immune system is well equipped to combat influenza, the virus encodes for proteins that specifically counteract these responses. The best characterized of these is a non-structural protein, NS1, found only in infected cells. NS1 acts as an interferon antagonist by sequestering double-stranded RNA and interrupting production of IFN-β by preventing the activation of IRF3, IRF7, and NF-κB. It also blocks the downstream activation of interferon-induced genes by preventing dsRNA-mediated activation of protein kinase R (Geiss *et al.,* 2002; Fernandez-Sesma *et al.,* 2006). In infected bone marrow-derived dendritic cells (DCs), expression of NS1 is also associated with suboptimal DC maturation and a decrease in production of the chemokines required to prime a T_H1-mediated immune response. In this way NS1 not only suppresses the innate

immune response, but can also downregulate adaptive immunity (Legge & Braciale, 2006; Fernandez-Sesma *et al.,* 2006).

In 2001 a new protein encoded by an alternate reading frame within the PB1 gene segment was discovered, and has also been implicated in the pathogenicity of influenza virus. The PB1-F2 protein, encoded by some influenza A viruses, specifically targets alveolar macrophages but not epithelial cells. This protein localizes to the mitochondria of infected alveolar macrophages where it causes apoptosis. Like NS1, PB1-F2 suppresses the immune response but is unique in that it specifically targets cells that are key mediators between innate and adaptive immunity (Coleman, 2007; Chen *et al.,* 2001).

3.3. Vaccines and Antivirals

Currently administered in the annual influenza vaccination campaign is a trivalent inactivated vaccine containing 15 μg of purified HA from three influenza viruses – an H3N2 and H1N1 influenza A virus and an influenza B virus (CDC, 2009). However, due to the variation in circulating viruses, the vaccine must be reformulated each year in order to provide efficient protection against strains predicted to circulate in the upcoming influenza season, and predictions are not always correct. An intranasally administered, live virus vaccine is also available and appear to be more effective, but is only approved for those aged 2-49 years, and is not safe for people in certain risk groups such as asthmatics and other immunocompromised individuals (Katz *et al.,* 2006; CDC, 2009).

In addition to vaccines aimed at preventing disease, two major classes of compounds are approved for use as prophylactic and therapeutic agents in North

America, each presenting a blockade at different points in the infectious cycle. The first group of compounds, consisting of amantadine hydrochloride and rimantadine, block the M2 ion channel, preventing virion acidification. The second type of anti-influenza compounds are the neuraminidase inhibitors, Zanamivir and Oseltamivir (Tamiflu®), which act by competing for the neuraminidase active site, preventing sialidase activity and inhibiting release of virions from infected cells. High levels of resistance to the M2 inhibitors has limited their use in recent years, however only a low level of resistance to the neuraminidase inhibitors has been observed clinically, making them the current line of defense against a epidemic and pandemic influenza (Wright *et al.,* 2006).

3.4. Antigenic Shift and Drift: Escape from Antibody-Mediated Detection

In response to infection with influenza virus, the humoral immune response primarily targets the surface glycoproteins, HA and NA. A great deal of variation exists among these glycoproteins, not only between but also within subtypes. A total of five antigenic sites are located on the distal portion of the HA molecule, and the nature of these sites dictates the HA type designation for a given virus. Similarly, the distal surface of NA molecules contains four antigenic sites made of multiple epitopes (Palese & Shaw, 2006). Sixteen HA and 9 NA subtypes have been found in nature, each being antigenically distinct from one another. Due to the segmented nature of the genome, a simultaneous infection with two influenza viruses can result in genetic reassortment, where new progeny virions contain a combination of genes from the two parent viruses. This phenomenon is termed antigenic shift, referring to a rapid change in the antigenicity of an infecting isolate after a virus emerges with an HA subtype different from that of the

circulating virus (Subbarao *et al.*, 2006; Wright *et al.*, 2006). Since the population is immunologically naïve to this virus, a pandemic can result. Another means of escape from immune recognition employed by influenza virus involves a slow and steady change in the antigenic nature of the virus. The lack of proof-reading by the RNA polymerase makes influenza virus highly prone to mutations, and when mutations occur in the antigenic regions of the glycoproteins it leads to a phenomenon termed antigenic drift, referring to the gradual accumulation of changes in antigenicity that ultimately create escape mutants not recognized by a previously exposed individual (Subbarao *et al.*, 2006). In addition to amino acid substitutions, influenza virus also changes glycosylation patterns to avoid detection. The hemagglutinin glycoproteins of avian viruses contain four glycosylation sites that can mask antigenic epitopes, and human viruses have up to five additional sites that allow them to escape antibody-mediated neutralization (Taubenberger *et al.*, 2000).

3.5. History and Pandemics

Descriptions of influenza-like disease causing outbreaks in people date back more than 2000 years. From the first reports of an outbreak by Hippocrates in 412 BC to present day infections by H5N1, influenza has plagued the human population, causing tens of millions of deaths and immeasurable levels of morbidity. Historical data indicates that several influenza pandemics likely occurred in the last two millennia, but the three pandemics of the 20th century are best documented and clearly attributed to influenza A virus.

The most infamous and by far the most devastating influenza pandemic occurred from 1918 to 1919. This outbreak occurred in two major waves; the first causing relatively mild disease in the spring of 1918, while the second wave was associated with excessive morbidity and mortality rates up to 25 times higher than normal, leading to an estimated 20-50 million deaths (Tumpey *et al.,* 2005; Reid *et al.,* 2001). A large proportion of deaths were due to secondary bacterial infections, however, hundreds of autopsies revealed severe pulmonary hemorrhage in patients who died within days of becoming ill; something not seen in previous epidemics. Additionally, the age-specific mortality rates were highly unusual in the 1918-1919 pandemic. Unlike typical epidemics displaying a U-shaped pattern of deaths by age, with highest rates in the elderly and very young, the 1918 trend pandemic had a W-shaped pattern, containing a large peak in the 15-45 year old category (Reid *et al.,* 2001).

The unprecedented severity of the pandemic has evoked major interest in the scientific community ever since it swept the globe, and the virus was recently reconstructed using PCR-amplified sequences obtained from formalin-fixed tissues from soldiers who succumbed to the disease, as well as from tissues obtained after excavating a woman buried in the Alaskan permafrost in November of 1918 (Taubenberger & Reid, 2002). The 1918 pandemic, or "Spanish flu" as it came to be known, was caused by the introduction of a novel H1N1 virus into the human population. Comparisons of the viral genes with other published influenza sequences indicate the virus is of avian origin, transmitted directly from the reservoir species to humans a short time before the pandemic (Taubenberger *et al.,* 2005). Progeny of the 1918 virus continued to cause annual epidemics of influenza for nearly forty years until a new subtype replaced it in the

late 1950s. In 1977 human H1N1 viruses reappeared during the "Russian flu", likely due to the accidental release of the virus from a laboratory. Pre-existing immunity in adults above the age of 25 accounted for the low degree of morbidity and mortality associated with the virus, whose descendents continue to circulate in the human population to date (Wright *et al.,* 2006; Potter, 1998).

The year 1957 brought the second pandemic of the 20th century, now referred to as the "Asian flu", which lead to approximately 1 million deaths worldwide by the end of the pandemic in 1958. The virus responsible contained new hemagglutinin (H2) and neuraminidase (N2) subtypes as well as a new PB1 gene, but retained the five other genes from the circulating strains of influenza. This indicates that an avian H2N2 virus reassorted with a human virus to produce the new isolate, and this reassortment may have occurred in pigs (Potter, 1998; Wright *et al.,* 2006).

The last major pandemic of the 1900s began in China in 1968 and was caused by a virus bearing a new HA (H3) subtype. This H3N2 virus, known as the "Hong Kong flu", also acquired a new PB1 from an avian virus and retained all other genes from the circulating descendants of the 1957 virus. The retention of the N2 segment from the circulating human viruses led to some degree of immunological protection from the new virus and mortality rates were therefore much lower than in previous pandemics. The Hong Kong H3N2 virus completely replaced the previous H2N2 human virus, and its descendants continue to circulate and cause annual influenza epidemics (Wright *et al.,* 2006; Potter, 1998).

In mid-February of 2009 an outbreak of influenza-like illness was reported in Veracruz, Mexico, and by early May the virus had spread to individuals in the United

States and Canada. The causative agent was isolated and determined to be a reassortant H1N1 influenza virus of swine origin, and on June 11th 2009 the WHO declared a pandemic was in progress (Neumann *et al.*, 2009, Maines *et al.*, 2009). As of July 6th, 2009, more than 94,000 laboratory-confirmed cases of pandemic H1N1 had been reported across 120 countries spanning all geographic locations (WHO, 2009b). The factors that allow a virus to cross the species barrier and become pandemic are not fully understood, and the current situation provides an opportunity to monitor the virus and its genetic changes as it spreads, enabling the scientific community to correlate specific mutations with increased virulence or transmissibility in real time. The current pandemic further highlights the need for surveillance systems and models that may better predict whether a given virus may have pandemic potential.

4. Ecology and Epidemiology

4.1. Avian Influenza

Of the five genera of *Orthomyxoviridae,* influenza A virus is the only genus to infect avian species. Waterfowl of the orders *Anseriformes, Passeriformes*, and *Charadriiformes* are considered the primary reservoirs of all influenza A viruses, and isolates of all known subtypes have been found in these populations (Alexander, 2007). Surveillance studies have isolated viruses from over 100 bird species representing 26 families, and this number will likely increase as further investigations are carried out. Poultry and other gallinaceous birds such as turkeys and quail are not among the natural reservoirs of influenza A viruses, but can be infected by viruses transmitted from wild birds.

In the wild, viruses are transmitted from bird to bird via the fecal oral route in contaminated water basins. There, viruses can remain viable for four days at 22°C and can even persist over winter in very cold climates, re-infecting birds in the spring (Alexander, 2007). However, some H5N1 isolates that are lethal for ducks have been recently found to replicate in both the gastrointestinal tract and the respiratory tract, indicating that transmission via aerosols may be involved on top of the traditional fecal-oral route of spread (Sturm-Ramirez *et al.,* 2004). Conversely, the respiratory route appears to be responsible for transmission of influenza in land birds such as domestic poultry. Here, direct contact with wild birds or their fecal material leads to the primary introduction of viruses into the flocks; however humans also play a role in the transmission of influenza viruses in domestic poultry. Objects contaminated with infected fecal material, in which the virus may survive for over 44 days, are easily spread within and between farms by the movement of farm workers and their vehicles, and are attributed to the secondary spread of virus between flocks (Capua & Alexander, 2008; Webster, 2006).

Avian influenza viruses are divided into two groups based on their resulting clinical disease in birds. Viruses of all 16 HA and 9 NA subtypes cause low pathogenic avian influenza, characterized by a mild or asymptomatic gastrointestinal infection in birds, whereas some viruses of the H5 and H7 subtypes cause highly pathogenic avian influenza (HPAI). HPAI is associated with a multisystemic infection and rapid mortality, resulting in up to 100% fatality rates in infected poultry flocks. At the molecular level, the pathogenicity index of a given virus is determined by the sequence at the cleavage site of the hemagglutinin precursor, HA0, which requires cleavage by host proteases into

HA1 and HA2 in order for the virus to be infectious. Low pathogenic avian influenza viruses contain a single arginine at the cleavage site, restricting cleavage to a limited number of extracellular host proteases. This limits the tissue tropism of the virus, as productive infection can only occur in anatomical sites where such proteases are found. Conversely, highly pathogenic viruses are characterized as containing multiple basic amino acids at the HA0 cleavage site. This permits cleavage by ubiquitous intracellular proteases and ultimately leads to systemic spread of the virus (Capua & Alexander, 2008; Taubenberger, 1998).

Highly pathogenic viruses emerge after their low pathogenic precursors are introduced into domestic poultry flocks from wild birds. Once in these new populations, viruses acquire the highly pathogenic cleavage site by one of two means. In most cases the multibasic amino acid cleavage site is acquired by stuttering of the polymerase during replication; however rare cases of non-homologous recombination with other gene segments have been recorded (Webster, 2006). Such was the case of a highly pathogenic outbreak of H7N3 influenza in British Columbia in 2004, where the insertion of 21 nucleotides from the matrix gene into the HA0 cleavage site generated a virus that wiped out entire flocks within days and led to the culling of 19 million birds in the Fraser Valley (Hirst *et al.,* 2004; Pasick *et al.,* 2005).

Since the first descriptions of 'fowl plague' in the 1878, highly pathogenic avian influenza viruses have caused devastating outbreaks in domestic poultry leading to the death and culling of hundreds of millions of birds. Twenty-four outbreaks involving high path H5 or H7 subtypes have occurred since 1959, and the incidence and severity have increased over time (Alexander, 2007; Capua & Alexander, 2008). The ongoing H5N1

epizootoc, reported in 62 countries as of February 2009, is unprecedented in its severity, longevity, and impact (OIE, 2009). In 1996 an H5N1 virus, A/Goose/Guangdong/1/96 (Gs/Gd/96), was reported in Guangdong, China that killed 40% of geese in the infected flock. Following that outbreak, Gs/Gd/96-like viruses continued to circulate and were responsible for human deaths in Hong Kong in 1997. Reassortment events between the Gs/Gd/96-like viruses and others from wild aquatic birds led to the establishment of several genotypes of H5N1 viruses in Asia, with the Z genotype becoming dominant. In 2002 there was marked antigenic drift in the HA and NA of the circulating H5N1 viruses in Asia, which resulted in new outbreaks of highly pathogenic influenza in domestic poultry. Of great concern was the fact that highly pathogenic viruses were then discovered to cause fatal infection for ducks and other aquatic birds, a highly unusual characteristic for an influenza virus. Since that time highly pathogenic viruses have caused further outbreaks in wild birds and in gallinaceous birds, and H5N1 appears to be endemic in Asia (Webster, 2006). The frequent interactions between wild aquatic birds and domestic poultry is likely contributing to a new directional flow of influenza viruses, whereby isolates that have mutated to highly pathogenic forms in domestic flocks are transmitted back to wild birds, which can asymptomatically carry the viruses and transmit them to other wild species that succumb to infection (Sturm-Ramirez *et al.,* 2004). This was most recently demonstrated in 2006, during an outbreak at the Qinghai Lake Nature Reserve in China, where over 6,000 wild birds died from HPAI. Molecular analysis of the causative agent revealed a reassortant virus bearing a combination of genes from two different chicken H5N1 highly pathogenic viruses (Wright *et al.,* 2006). In addition to the vast numbers killed by infection, hundreds of millions of birds have been culled as a

result of the H5N1 epizootic, and 256 of the 409 documented human cases since 2003 have been fatal (Webster, 2006; WHO, 2009a).

4.2. Receptor Specificity and Interspecies Transmission

In their natural hosts influenza viruses are in a state of evolutionary stasis, with much lower mutation rates compared to viruses circulating in mammals. Once introduced into alternative hosts, viruses experience rapid evolution and often develop mutations that increase their infectivity and transmissibility in a new host (Webster, 2006; Capua & Alexander, 2008). In addition to transmission from wild birds into poultry, avian viruses have also been transmitted directly to other species, including felids, canines, whales, seals, horses, pigs and humans. Such transmission events have been infrequent, but have led to epizootics, enzootics, and pandemics (Wright *et al.*, 2006; Van Reeth, 2007).

In order for a virus to successfully cross the species barrier, several constraints at the virus and host levels must be overcome. The first step to viral infection is recognition of target cells and specific binding with viral receptors on the cell surface to facilitate entry, and this is governed by the interaction of the HA glycoprotein with these receptors. Influenza viruses recognize carbohydrates with a terminal sialic acid residue, however avian and mammalian viruses differ in their specificity for these molecules based on the type of terminal sialic acid linkage (Neumann & Kawaoka, 2006). The avian GI tract is predominated by α-2,3-linked sialic acid species, while the human respiratory tract contains mostly α-2,6-linked sialic acids, though α-2,3-linked sialic acid species are found on some ciliated cells in the lower respiratory tract. Accordingly, mammalian

viruses are specific for α-2,6-linked SA residues while avian viruses are specific for α-2,3-linkages. The presence of additional carbohydrates on the receptor molecule further influences receptor specificity, as viruses isolated from wild waterfowl have a low affinity for molecules with complex carbohydrates while those isolated from domestic poultry and mammals have a higher affinity for such molecules (Gambaryan *et al.,* 2002).

Even though avian viruses can infect cells deep within the human respiratory tract, a change in affinity of their HA towards the mammalian receptor conformation is required for the virus to successfully transmit from human-to-human, since influenza viruses are transmitted via droplets and aerosols released from the upper respiratory tract. Additionally, NA must be able to interact with the receptor to cleave SA residues from nascent HA molecules during the budding process. Therefore, both the HA and NA glycoproteins must shift their affinities from the avian to mammalian receptor for efficient transmission in the human population (Neumann & Kawaoka, 2006).

4.3. Influenza and Pigs

Pigs are among the few species of mammals in which specific lineages of influenza A viruses have become established and continually circulate, causing enzootic disease. Swine influenza virus (SIV) of the H1N1, H1N2, and H3N2 subtypes causes a primarily respiratory disease of notable morbidity but very low mortality in swine populations. Infection is highly localized to the bronchial and bronchiolar epithelial cells, though interstitial and alveolar macrophages are also infected (Jung *et al.*, 2002). Symptoms appear after a 1-3 day incubation period and include labored breathing, inappetence, weight loss, inactivity, fever, coughing, and sneezing; with most pigs in a

herd becoming infected at the same time. Other complications may include reproductive and neonatal problems (Easterday & Hinshaw, 1992).

Respiratory secretions from pigs experimentally infected with SIV contain proinflammatory cytokines including TNF-a, type I IFNs, and IL-6, and peak titers are associated with peak viral titers and clinical severity (Charley *et al.,* 2006). The adaptive immune response to influenza is much the same as in humans, involving neutralizing IgA and IgG responses and CTL-mediated killing of infected cells (Charley *et al.,* 2006).

Experimental infections of pigs with influenza viruses showed that alveolar macrophages in particular were essential for recovery from H1N1 SIV infection, and this most likely due to TNF-α and IFN-γ production. Interestingly, swine alveolar macrophages infected with different influenza A subtypes produced different amounts of TNF-α, with a greatly intensified response to an H3N2 than an H1N1 virus. This was demonstrated both *in vivo* and *in vitro*, and determined to be influenced by the nature of the HA and NA glycoproteins of the different viruses (Kim *et al.,* 2008; Seo *et al.,* 2004).

While the porcine immune response to influenza is similar to that seen in humans, there are a few notable differences. IL-1, another key inducer of the host inflammatory response, is also found at elevated levels in respiratory secretions from infected pigs, whereas no increase was noted in experimentally infected human volunteers (Van Reeth, 2000). Another difference between the swine and human immune response comes from experimental work with highly pathogenic H5N1 and the rescued 1918 pandemic influenza viruses. Unlike humans, mice, and other experimental models that present with hypercytokinemia after infection, pigs experience limited clinical disease in response to experimental infection with H5N1 or 1918 viruses (Weingartl *et al.,* 2009; Lipatov *et al.*

2008). Further, it has been difficult to detect cytokine up-regulation in these experimentally infected pigs (Weingartl *et al.,* unpublished data).

Historical data show that concurrent with human cases of influenza during the 1918 pandemic, similar clinical signs were observed in swine populations in the autumn of 1918 (Weingartl *et al.,* 2009, Taubenberger *et al.,* 2001). This situation repeated itself in 1968, when pigs showed symptoms of influenza following the onset of the human H3N2 pandemic (Easterday & Hinshaw, 1992). Comparative analyses of A/Swine/Iowa/15/1930 (H1N1), a descendent of the original SIV virus, and the recently reconstructed 1918 H1N1 pandemic virus confirmed that the two are genetically related. Since that time, a number of interspecies transmissions of influenza viruses between humans and pigs have been documented. In 1976 several hundred military personnel came down with flu-like illness, and the virus isolated from patients, A/NJ/8/76 (H1N1) was highly related to a swine flu isolate. Isolated incidents of swine to human transmission have occurred on a number of occasions since that time; however these viruses were not successfully maintained in the human population (Easterday & Hinshaw, 1992; Van Reeth 2007). Though there is no evidence to implicate pigs in the generation of the previous pandemic viruses, the 2009 pandemic has highlighted the role of pigs in the generation of a pandemic virus, which has resulted for the reassortment of three swine influenza viruses (Neumann *et al.*, 2009).

Pigs are susceptible not only to swine and occasionally human influenza viruses, but also to avian influenza viruses. Cells lining the swine respiratory tract contain sialic acid species with both α-2,6 and α-2,3 linkages to galactose molecules, making them susceptible to infection by both human and avian influenza viruses (Ito *et al.*, 1998). This

biological property led to the hypothesis that pigs may serve as a mixing vessel for the generation of reassortant human-avian influenza A viruses with pandemic potential. This theory is supported by the fact that most SIV are reassortants containing genes from human, swine, and avian viruses (Van Reeth, 2007). Another possible role for pigs in the generation of pandemic influenza is allowing avian viruses to shift receptor affinity towards the human receptor. Continued replication of avian viruses in pigs leads to a shift in receptor affinity from α-2,3 to α-2,6, as seen in avian-like swine viruses analyzed over time from their initial appearance in pigs (Ito *et al.*, 1998). The role of pigs in the transmission of highly pathogenic H5N1 viruses to humans must not be overlooked in the future. At present, the circulating highly pathogenic Asian H5N1 viruses have low replication fitness in experimentally infected pigs, and sero-epidemiological analysis of pig sera from Vietnam during the 2004 poultry isolates showed only 0.25% of pigs had antibodies to H5N1 (Lipatov *et al.*, 2008; Van Reeth, 2007). While H5N1 viruses are not stably maintained in the pig population as of yet, ongoing surveillance in swine is critical for pandemic preparedness.

4.4. PB2

While the viral glycoproteins play a pivotal role in determining host range of influenza viruses, the internal proteins must also function in the new host environment in order for efficient genome transcription and replication to occur. As part of the trimeric viral polymerase complex, the central role of PB2 is to recognize and bind to capped host cellular pre-mRNA transcripts to serve as primers for viral transcription (Shi *et al.*, 1995). PB2 has been implicated in host range restriction of influenza since the late 1970's, when

it was noted that this protein restricted the growth of certain avian viruses in mammalian cells (Subbarao *et al.*, 1993). Since that time, residue 627 of PB2 has been implicated as the major determinant of host restriction and tissue tropism in mice. Most avian viruses have glutamic acid at this position, restricting growth in mammalian cells, whereas mammalian viruses have lysine at this position, allowing for successful replication in mammalian systems (Noah & Krug, 2005; Labadie *et al.*, 2007). Two theories have been proposed for the role of this residue in host range restriction. The first suggests that it directly interacts with host cell factors required for RNA transcription and replication, and differences between factors from avian and mammalian species require a specific amino acid for optimal interaction (Noah & Krug, 2005; Tarendeau *et al.*, 2008). The second hypothesis is that residue 627 is a determinant of temperature sensitivity, allowing for optimal RNA polymerase activity at the temperature of the natural host. The core temperature or the avian GI tract, the site of avian influenza virus infection, is approximately 41°C, whereas temperatures in the human respiratory tract, the site of human influenza virus infection, range from 33°C in the upper portion to 37°C in the lower respiratory tract. Accordingly, glutamic acid is thought to confer high temperature preference to avian viruses, while lysine in mammalian viruses allows for improved polymerase activity at lower temperatures (Noah *et al.*, 2005, Massin *et al.*, 2001, Bradel-Tretheway *et al.*, 2008). This was first demonstrated experimentally by comparing growth of avian and human viruses in MDCK cells, where titers of avian viruses were significantly decreased at 33°C incubation temperature compared to 37°C while human viruses reached similar titers at both temperatures (Massin *et al.*, 2001). Several investigators have since demonstrated its role by looking at whole virus replication or

transcription/replication activity of *in vitro*-reconstituted RNP complexes in mammalian cell lines, including primary human cells of respiratory origin. However, a recent publication demonstrated that a reconstituted polymerase complex from an avian-derived virus with a lysine at PB2 residue 627 retained activity over a wide range of temperatures (Bradel-Tretheway *et al.,* 2008). Additionally, the nature of PB2 does not seem to have an influence on temperature-dependent activity in avian cells, as polymerase function from viruses possessing either a lysine or glutamic acid is maintained over a broad range of temperatures (Hatta *et al.,* 2007, Bradel-Tretheway *et al.,* 2008, Labadie *et al.,* 2007). This suggests that the effects of PB2 may be system specific, and that residue 627 is not always an accurate prediction of polymerase function at varied temperatures.

Gaps in Knowledge

The full repertoire of viral and host factors that allow influenza virus to successfully cross the species barrier remains largely unknown, but research has pointed to a number of viral characteristics associated with adaptation to a new host. One such factor is the ability of a virus to successfully replicate under the physiological conditions of its new host. With the avian resting temperature sitting several degrees higher than that of the human respiratory tract, it is reasonable to expect that most avian viruses would have difficulty replicating at the cooler temperatures of the human respiratory tract. While some studies have demonstrated this, it is necessary to screen a large number of avian viruses to see whether this expectation holds true. Further, if certain viruses are found to be an exception to the rule it is important to look for any distinguishable genetic traits that may be responsible. Several investigators have pointed

to the role of the PB2 gene in determining temperature sensitivity of influenza viruses, with residue 627 playing a key role. However, many of these studies have had system specific results. At present, no data exists regarding viral temperature sensitivity in a swine system. Given the important role of pigs in the interspecies transmission of influenza virus, such information would be of great importance. As pigs are naturally infected with swine, human, and avian influenza viruses, they present an ideal model within which to study the nature of these viruses.

The immune response to viral infection presents another area of influenza virology where host-virus interactions are incompletely understood. The fact that influenza viruses differ in the degree of cytokine response induced has important clinical implications. On one hand, the appropriate cytokines must be produced and secreted by infected cells and the sentinel immune cells in the pulmonary system to effectively clear the infection. However, if this response is dysregulated; either by a lack of immune recognition or an overly exaggerated reaction, severe disease may result. Comparative investigations on responses generated against different viruses in a natural host model are required to further elucidate the viral factors with an influential role on the host immune response. Since the swine and human responses to the same influenza isolates can vary so greatly, it is important to understand the underlying factors that allow pigs to escape severe disease from viruses with highly pathogenic characteristics in other species.

5. Research Objectives

Hypothesis 1) An immortalized swine alveolar macrophage cell line would serve as an appropriate *in vitro* model to study influenza A viruses of avian and mammalian origin.

Objective: To find a suitable *in vitro* model to determine the dynamics of influenza viruses from various host backgrounds, including human, avian, and swine.

Hypothesis 2) Influenza A viruses would grow at temperatures most closely representing those of their natural hosts; avian viruses would grow best at the avian core temperature (41°C) and have poor growth at lower human temperatures (33°C), while human viruses would thrive at the temperatures of their natural host (33°C - 37°C) but would not replicate well at the higher avian temperature.

Objective: To use the *in vitro* model to study the effects of different physiologically relevant growth temperatures on the viability of a wide variety of human, swine, and avian influenza viruses, including some well characterized and high profile isolates along with others obtained from surveillance studies.

Hypothesis 3) The sequence of the PB2 genes from experimental isolates, especially at amino acid position 627, would determine the fitness of each virus at different temperatures and correlate to experimental results. Avian viruses that replicate to highest titers in the warmest test conditions would contain avian-associated PB2 amino acids while human viruses that replicate to highest titers in the cooler test conditions would contain human-characteristic PB2 sequences.

Objective: To determine whether differences in temperature preference could be attributable to the PB2 genes of each experimental isolate, with specific focus on amino acids indicated in the literature to play an important role in temperature sensitivity.

Hypotheses 4i) Influenza A isolates of different subtypes and different host backgrounds would vary in the degree of cytokine response they induced.

ii) Differences observed in cytokine induction would be attributable to the HA and NA glycoproteins of the different virus isolates.

Objective: To determine whether different influenza isolates induced different levels of cytokines following infection of the immortalized porcine alveolar macrophage cell line, and if these differences could be attributed to specific viral proteins.

Materials & Methods

1. Cells

Mandin-Darby Canine Kidney (MDCK) cells, obtained from the American Type Culture Collection (ATCC), were maintained in Alpha Modification Eagle's Medium (AMEM) (Wisent, Cat No. 310-011 CL) with 10% Fetal Bovine Serum (FBS) (Wisent). Immortalized porcine alveolar macrophage cells (IPAMs, clone 3D4/31) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent, Cat No. 319-005CL) with 10% FBS, 1% Sodium Bicarbonate (Sigma, Cat No. S8761) and 1% non-essential amino acids (Sigma, Cat No. M7145). 293T cells, obtained from ATCC, were maintained in DMEM supplemented with 10% FBS. All cells were kept at 37°C in a 5% CO_2 incubator for routine passaging. Cells were maintained in 75 cm^2 culture flasks (Corning) and passaged every 3-4 days as necessary by washing once with PBS and trypsinizing with 0.25% trypsin-EDTA (Gibco, Cat No. 25200).

2. Cell Characterization

2.1. Confocal Microscopy

To determine whether both avian and human cell-associated sialic acid linkages were present on the cell surface of IPAMs, cells were stained with fluorescently labelled lectins for detection of α-2,3 and α-2,6-linked SA residues. IPAMs were seeded on sterile glass histoslides (Fisher, Cat No.15-188-52) 24 hours prior to staining. Slides were then washed in PBS-filled coplin jars, fixed in ice-cold acetone for 15 minutes, and air-dried for 15 minutes. Five slides were included for analysis: 1. Unstained negative control; 2. Cells stained for presence of α-2,6-linked receptors using biotinylated

Maackia Amurensis Lectin II (MAA) (Vector Laboratories, Cat No. B-1265) and Texas Red-Avidin D (Vector Laboratories, Cat No. A-2006) as a secondary stain; 3. Cells stained for presence of α-2,3-linked receptors using Fluorescein Elderberry bark Lectin (SNA) (Vector Laboratories, Cat No. FL-1301); 4. Cells stained with only the secondary Texas red-Avidin D; 5. Double stained cells for detection of α-2,3 and α-2,6-linked receptors. Additional work by collaborating scientists at the National Centre for Foreign Animal Disease showed the specificity of the immunofluorescent lectins when used to detect sialic acid linkages on duck tissues (Berhane Y, unpublished data).

The staining procedure was carried out in two steps. First, slides stained for detection of α-2,6-linked receptors were treated with 500 µl of biotynilated MAA prepared at a concentration of 20 µg/ml in 0.1% PBS-Tween (PBS with 0.1% v/v Tween 20) (Sigma, Cat No. P1379), while other slides received PBS-Tween only. Slides were incubated at room temperature for one hour, and then washed three times in PBS-Tween with shaking for 10 minutes per wash. In the second step, fluorescently labeled reagents (Fluorescein-SNA and Texas red Avidin-D) were prepared at concentrations of 20 µg/ml in PBS-Tween, and 500 µl of the resultant suspension was added to the appropriate slides. For the double-stained slide, the fluorescently labeled reagents were prepared together in a tube of PBS-Tween so the resultant 500 µl suspension contained the proper amount of each dye. The control slide was treated with 500 µl of PBS-Tween to account for any background fluorescence not attributed to staining. All slides were incubated at room temperature for one hour in the dark and then washed 3X in PBS-Tween as performed previously. After air-drying in the dark, one drop of Antifade reagent (Invitrogen, Cat No. S2828) was added to the slides, which were then sealed and stored in

the dark until viewing. Fluorescence was analyzed on an Olympus Fluoview FV300/500/1000c confocal microscope.

2.2. Flow Cytometry

IPAMs were analyzed for the surface expression of monocyte and macrophage-specific surface markers by flow cytometry. Confluent monolayers of IPAMs were washed twice with PBS (without calcium chloride and magnesium chloride), trypsinized with 0.25% trypsin-EDTA, resuspended in serum-free media and counted. The appropriate volume containing $1.0x10^6$ cells was added to a sterile 1.5 ml microcentrifuge tube (Diamed, Cat No. PRE150-N) and left to incubate for 20 minutes at room temperature. Cells were then resuspended in100 µl of PBS (calcium chloride and magnesium chloride-free) containing 0.1% w/v sodium azide and spun for two minutes at 1,000 revolutions per minute (RPM) at 4°C. Supernatant was discarded and the cell pellet was resuspended in 100 µl of primary antibody (prepared in PBS-sodium azide) and incubated at 4°C in the dark for 45 minutes. Cells were washed twice with 100 µl PBS-sodium azide, with a two-minute spin at 110 x g following each wash, and then resuspended in 100 µl of secondary antibody (prepared in PBS-sodium azide), or in PBS-sodium azide alone if no secondary antibody was required. Cells were incubated at 4°C for 45 minutes in the dark, washed twice as described above, and resuspended in 1ml PBS for analysis. Samples were analyzed on a Beckman Coulter FC500 flow cytometer. Cells were gated based on their forward and side-scatter properties, measuring size and granularity, respectively, and then evaluated for the presence of specific surface molecules. IPAMs were stained for detection of the following CD molecules on their

surface: SWC3a, a porcine pan-myeloid marker present on nearly 70% of adherent PBMCs; CD14, a marker expressed highly by monocytes but downregulated upon maturation to macrophages; and SWC9, a marker not expressed by monocytes but upregulated upon macrophage differentiation (McCullough *et al.,* 1999). Primary antibodies conjugated directly to fluorescent dyes were utilized to detect these molecules and included Mouse Anti-Porcine Monocyte/Granulocyte SWC3a:PE (Beckman Coulter, Cat No. 732793) and Mouse Anti-Porcine SWC9:FITC (Serotec, Cat no. MCA1973F). The primary antibody Mouse anti-Porcine CD14 (Serotec, Cat no. MCA1218) was also used but required the addition of a fluorescently labelled secondary antibody, and an Alexa Fluor 488 conjugated Rabbit anti-Mouse antibody (Molecular Probes, Cat No A11059) was used. Unstained cells were included as negative controls.

3. Viruses

Ten viruses, including four from human, two from swine, and four from avian hosts were included for analysis. A complete list of viruses used along with a brief description of their epidemiological background is found in Table 2. All avian viruses were provided by Dr. John Pasick at the National Centre for Foreign Animal Disease. Human viruses, excluding the SouthCarolina/1918 virus, were obtained from ATCC, and the Swine/Texas virus was obtained from Dr. Kelly Lager at the USDA, Ames, Iowa. Plasmids encoding the eight segments of the Swine/Iowa virus were provided by Dr. Jürgen Richt, and the virus was previously rescued in house in Containment Level 3 (CL-3) using an eight-plasmid reverse genetics system as described by Hoffmann and colleagues (Hoffmann *et al.,* 2002). The SouthCarolina/1918 human virus was also

previously rescued in house in the CL-4 lab using the 12-plasmid system described by Fodor and colleagues (Fodor *et al.,* 1999), which was provided by Dr. Adolfo Garcia-Sastre from the Mount Sinai School of Medicine. The 1930:1918 HANA virus was previously rescued using a combination of plasmids from both rescue systems. All viruses were handled in the CL-3 laboratory at the National Centre for Foreign Animal Disease, CFIA except for Chicken/Vietnam, which was handled in an enhanced CL-3 lab (CL-3+), and the SouthCarolina/1918 and recombinant 1930:1918HANA rescued viruses, which were handled exclusively in CL-4. Working stocks of virus were generated by egg inoculations, except for the rescued SouthCarolina/1918 virus and the 1930:1918HANA rescued viruses, which were generated in tissue culture.

4. Egg Infections

Influenza virus isolates were inoculated into the allantoic cavity of 9 day-old specific pathogen-free embryonated hen's eggs, which were monitored daily for viability following inoculation. Inoculum was prepared by diluting virus 100- to 10,000-fold (depending on pathogenicity level) in sterile PBS and filtering through a 0.22 micron Millex Syringe Driven Filter Unit (Millipore, Cat No. SLGV033NS). Prior to inoculation, eggs were cleaned with iodine and then injected with 200 µl of virus inoculum using a 23G1 PrecisionGlide needle (Becton Dickinson, Cat No. 305145). Following infection, dead eggs were refrigerated at 4°C for a minimum of one hour before harvesting allantoic fluid. Following harvest, allantoic fluid (AF) was clarified by centrifugation at 2,500 x g for 25 minutes. Clarified fluid was filtered using a 0.22 micron filter, and 1.0 ml aliquots were stored at -80°C for further use.

Table 2. Full names and abbreviations, species of isolation and epidemiological information of the ten influenza viruses used for experimental purposes.

Name	Sub-type	Designation	Origin	Epidemiological Information
A/South Carolina/1918	H1N1	SouthCarolina/ 1918	Human	Reconstructed virus using sequences isolated from casualties of the 1918 pandemic. Likely caused by the introduction of an avian virus into the human population (Wright *et al.*, 2006).
A/Wilson-Smith/33	H1N1	WS/33	Human	First human influenza virus isolated. Descendent of 1918 virus (Wright *et al.*, 2006).
A/Puerto Rico/8/34	H1N1	PR/8	Human	Widely used lab strain of influenza (Cheung & Poon, 2007).
A/Hong Kong/8/68	H3N2	HongKong/68	Human	Causative agent of the third major pandemic of the 20th century. Virus emerged after previously circulating human strain recombined with avian isolates and new HA and PB1 genes were introduced (Wright *et al.*, 2006).
A/Swine/Iowa/15/1930	H1N1	Swine/1930	Swine	First influenza virus isolated. A descendent of the 1918 virus following introduction into the swine population (Weingartl *et al.*, 2009).
A/Swine/Texas/4199-2/ 98	H3N2	Swine/Texas	Swine	A triple reassortant swine virus containing human-origin HA, NA and PB1 genes, swine-origin NS, NP and M gene, and avian virus-origin PB2 and PA genes (Vincent *et al.*, 2008).
A/Duck/Alberta/C-16/ 2007	H7N7	Duck/Alberta	Avian	Canadian wild bird isolate of low pathogenicity index. Isolated from ducks found dead over winter (Pasick *et al.*, unpublished data).
A/Chicken/British Columbia/514/2004	H7N3	Chicken/BC	Avian	Highly pathogenic virus responsible for 2004 Frasier Valley outbreak in poultry farms, led to the culling and death of nineteen million chickens (Wright *et al.*, 2006).
A/Emu/Texas/39924/98	H5N2	Emu/Texas	Avian	Highly pathogenic variant of an Emu isolate (Clavijo *et al.*,2002).
A/Chicken/Vietnam/14/ 2005	H5N1	Chicken/Vietnam	Avian	Highly pathogenic virus, immediate relative of isolates that caused the deaths of millions of domestic and wild birds as well as hundreds of humans in Asia (Pasick *et al.*, 2007).

5. Hemagglutination Assays

Hemagglutination assays (HAs) were used to determine the presence and relative amounts of viral particles in harvested allantoic fluid from infected eggs as well as tissue culture supernatants during time-course experiments. Fifty microlitres of Dulbecco's Phosphate Buffered Saline (PBS) was added to the required number of rows in 96-well, V-bottom assay plates (Sigma, Cat No. CLS3894). Fifty microlitres of harvested AF was added to the first well of a given row (now containing 100 µl including sample and PBS), and serially diluted across the row by transferring 50 µl from the first well into the second well and so on until the last well, from which 50 µl was removed and discarded so that all wells contained 50 µl of virus at successive two-fold dilutions. A row of PBS only was included on each assay plate to act as a negative control. Fifty microlitres of 0.5% chicken erythrocytes (prepared in sterile PBS) were then added to each well; plates were gently tapped to mix, and incubated for 25 minutes at room temperature. The HA titre was determined as the reciprocal of the highest dilution of sample where hemagglutination was observed.

6. Endpoint Titration of Stock Virus

The titres of all virus stock solutions were determined using the 50% tissue culture infectious dose assay ($TCID_{50}$). Confluent monolayers of MDCK cells on 96-well plates (Corning) were infected with serial ten-fold dilutions of virus stock, using eight replicate wells per dilution. The final two columns were mock-infected cell controls, and all $TCID_{50}$ assays were performed on duplicate plates. Fifty microlitres of inoculum, prepared in serum-free AMEM containing 1.0 µg/ml L-1-tosylamido-2-

phenylethyl chloromethyl ketone (TPCK) treated-trypsin (Sigma, Cat. No. T8802), were added to each well and plates were incubated at 37°C with 5% CO_2. After one hour of incubation, an additional 50 µl of AMEM containing 0.6% Bovine Serum Albumin (Sigma, Cat No. A8412) and 1.0 µg/ml TPCK trypsin was added to each well. Plates were incubated for 4-5 days at 37°C with 5% CO_2. CPE was visualized after cells were fixed for 20 minutes with 10% buffered formalin (Fisher) and stained with a 0.5% crystal violet solution prepared in deionized water. Virus titre was calculated using the method of Reed and Muench.

7. Time-course Experiments

In order to examine the replication fitness of different viruses at varied physiologically relevant temperatures, time-course experiments were carried out for a 48-hour period to compare replication at 37°C, 33°C, and 41°C. Confluent wells of IPAMs in 24-well tissue culture plates (Costar) were infected with influenza viruses and cultured at three different temperatures to reflect the human (33°C and 37°C) and avian (41°C) host conditions at the anatomical site where infection typically occurs. Two plates were used per experiment, with one incubated at 37°C and the other incubated at 33°C or 41°C. When three incubators were available, three plates were used per experiment, with one plate incubated at each of the three different temperatures (33°C, 37°C, and 41°C). One well per plate was trypsinized to determine the average number of cells per well, and this value was used to determine the amount of stock virus required for infection. Cells were washed once with PBS and then infected at a multiplicity of infection (MOI) of 0.07 $TCID_{50}$ per cell, allowing for multiple rounds of infection. Each well received 200 µl of

serum-free DMEM containing 0.1 μg/ml TPCK-Trypsin and the appropriate amount of stock virus, or was mock infected with virus-free media as described above. At one hour post-infection inoculum was removed and replaced with 1.0 ml DMEM containing 0.3% BSA and 0.1 μg/ml TPCK-Trypsin. At 1.5, 24, and 48 hours post-infection, 500 μl of supernatant was collected from four infected and one mock-infected well for RNA extraction (Figure 1). Fifty microlitres from the same wells were used for HAs to determine the presence of viral antigen, and the remaining supernatant from the sampled wells were harvested and frozen individually at -70°C for later analysis.

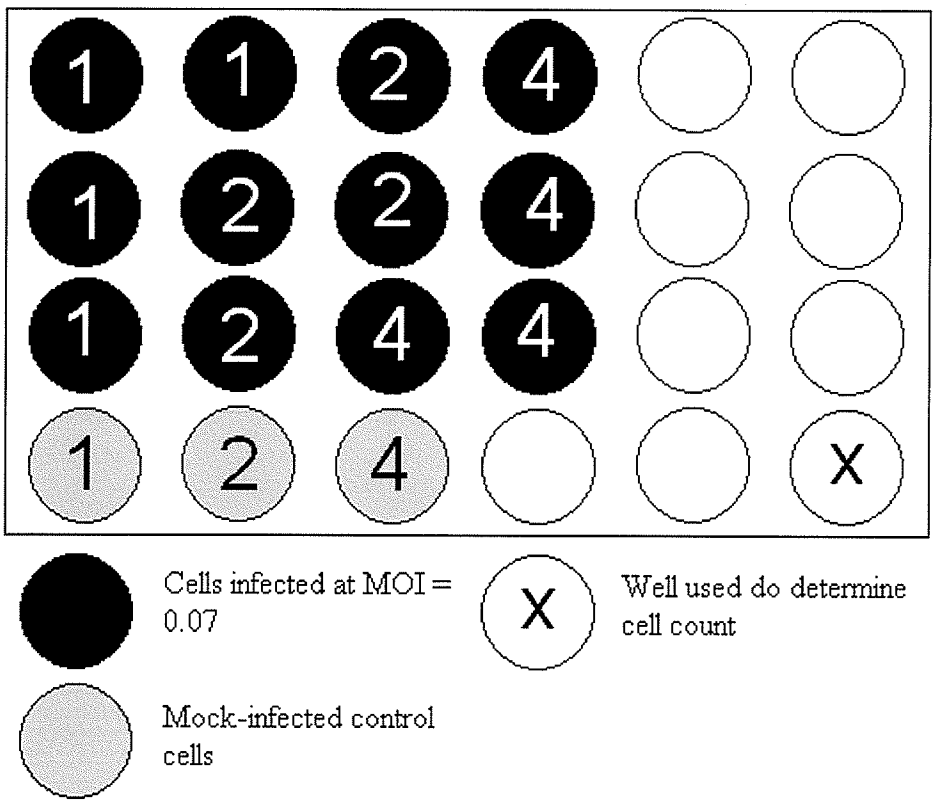


Figure 1. Experimental set-up for time-course infections of IPAMs with influenza viruses. Infected and mock-infected control cells were sampled at 1.5 (1), 24 (2) and 48 (4) hours post-infection.

8. Removal of Nucleic Acids from CL-4

Supernatants from time-course experiments conducted in the CL-4 laboratory were removed in RLT buffer (Qiagen) (See Appendix II for preparation instructions) for extraction of nucleic acids. Five hundred microlitres of experimental supernatant was added to a microcentrifuge tube containing 1.0 ml of RLT buffer, and left to sit at room temperature for 30 minutes. Tube contents were then transferred to a new microcentrifuge tube, which was then dunked out of CL-4 in a Saf-T-Pak (Pt No. 062) filled with a 5% Microchem Plus solution (NCL Inc, Product No. 0255). Saf-T-Paks were immersed in a 5% Microchem dunk tank for a minimum of 30 minutes prior to removal from the CL-3 side of the tank, and contents were opened inside a biosafety cabinet.

9. RNA Extraction

RNA from experimental supernatant was extracted using the Qiagen RNeasy Protect Mini Kit (Qiagen, Cat No.74126) (See Appendix I for preparation of buffers prior to extraction). Five hundred microlitres of sample were collected in nuclease-free microcentrifuge tubes containing 1.0 ml of RLT buffer. Samples were either extracted after 30 minutes, or stored at 4°C and extracted within 24 hours using a vacuum manifold as per manufacturer's specifications. Samples in RLT were added to Qiagen spin columns, washed with 700 µl of RW1 wash buffer, and then washed twice with 500 µl RPE wash buffer. Mini columns were then removed from manifold, placed into collection tubes, and spun at 20,800 x g for one minute. Collection tubes containing residual liquid were then discarded, and spin columns were placed into fresh collection

tubes. RNA was eluted with 40 µl of RNase-free water by spinning for one minute at 10,600 x g and frozen at -70°C until needed. Each extraction included experimental samples, a positive control (either stock virus or inoculum), and a negative PBS control.

10. Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Extracted RNA was analyzed via real time RT-PCR for detection of the Matrix gene. Each PCR run included experimental samples from all temperatures tested in a given time-course experiment. Master mixes were prepared using Qiagen One-Step RT-PCR kits (Qiagen, Cat No. 210210) and reactions were carried out under the following conditions:

<table>
<tr><th>Reagent</th><th>Volume (µl)</th><th>Reaction Concentration</th><th colspan="4">Cycle Parameters</th></tr>
<tr><td rowspan="2">RNase-free H_2O</td><td rowspan="2">6.95</td><td rowspan="2"></td><td>Stage 1
1X</td><td>Stage 2
1X</td><td colspan="2">Stage 3
45X</td></tr>
<tr><td>50°C</td><td>95°C</td><td>94°C</td><td>60°C</td></tr>
<tr><td>5X Buffer</td><td>5</td><td>1X</td><td>1800 seconds</td><td>900 seconds</td><td>1 second</td><td>20 seconds</td></tr>
<tr><td>25 mM $MgCl_2$ (Roche)</td><td>1.25</td><td>3.75 mM</td><td colspan="4" rowspan="8">Probe:
(5'/56FAM/TCAGGCCCCCTCAAAGCCGA/3BHQ_1/ - 3', Integrated DNA Technologies)

Primers:
Forward: (+25-5):
5' AGATGAGTCTTCTAACCGAGGTCG (Invitrogen)
Reverse: (-124-3):
5' TGCAAAAACATCTTCAAGTCTCTG (Invitrogen)</td></tr>
<tr><td>DNTPs</td><td>0.8</td><td>320 µM each</td></tr>
<tr><td>Forward Primer (20um)</td><td>0.5</td><td>0.4 µM</td></tr>
<tr><td>Reverse Primer (20 µm)</td><td>0.5</td><td>0.4 µM</td></tr>
<tr><td>Probe (6 µM)</td><td>0.5</td><td>0.12 µM</td></tr>
<tr><td>Anti-RNase (20U/µl) (Ambion)</td><td>0.5</td><td>10 U</td></tr>
<tr><td>OneStep enzyme mix</td><td>1</td><td></td></tr>
<tr><td>Template RNA</td><td>8</td><td></td></tr>
</table>

Reactions were carried out on a Cepheid Smart Cycler and analyzed with accompanying software (Version 2.0b). Each Smart Cycler run included experimental samples from all temperatures tested, positive and negative extraction controls, a no template control, and a dilution series of Matrix gene from plasmid DNA preparations in order to construct a standard curve. The plasmid control consisted of the SouthCarolina/1918 M gene

segment cloned into a pPol I vector and confirmed by automated DNA sequencing (described below). A detailed calculation for determining the number of matrix gene copies in the plasmid standards is found in Appendix II. Experimental samples were plotted against this curve in order to determine matrix copy number per sample.

11. Construction of Growth Curves

Results from real time RT-PCR were used to construct growth curves for each virus in order to compare replication rates (measured by relative amount of Matrix gene transcripts) at the three different temperatures over 48 hours. Data from three independent experiments were compiled, and the mean matrix copy number and corresponding standard deviation was plotted for 1.5, 24, and 48-hour post-infection sampling points for all viruses at 33°C, 37°C, and 41°C incubation temperatures.

12. Statistical Analyses

The difference in mean virus titres at the three different experimental temperatures, using real time RT-PCR results as readout, was analyzed for statistical significance with SPSS statistical analysis software, using a one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test. RT-PCR results from each sample were averaged to generate a mean value for each temperature in a given experiment. The ANOVA was conducted using mean data from each independent experiment so that 3 mean values at 33°C, 3 mean values at 41°C, and at least 3 mean values at 37°C were compared for each experimental isolate. The number of means at 37°C was dependent upon how many incubators were available during the experimental

procedures. The 37°C test condition was always included as a standard against which the other temperatures were compared. When only two incubators were available, experiments comparing growth at 37°C and 33°C were repeated three times, as were experiments comparing growth of 37°C and 41°C. In such situations, 3 mean data sets were generated for 33°C and 41°C real-time RT-PCR results, while 6 means were generated from 37°C experiments. When three CO_2 incubators were available, replication at all three experimental temperatures could be simultaneously compared so that a total of 3 mean data sets were generated for 37°C, 33°C, and 41°C test plates. In certain situations, the ANOVA was calculated on fewer than 3 means for a certain temperature if a particular trial did not accurately reflect growth patterns seen in the other trials. One such situation was if all infected supernatants had negative RT-PCR results at a particular sampling time during an experiment, but infected samples in repeat experiments gave positive results.

13. PB2 Gene Sequencing of Experimental Isolates

PB2 sequences of Swine/Iowa, South Carolina/1918, Chicken/BC, Chicken/Vietnam, and Duck/Alberta were previously determined by staff at the NCFAD lab, or in labs of collaborating investigators. The sequence of Emu/Texas PB2 gene was previously published, and confirmed by in-house sequencing reactions to possess the same amino acids as described in Genbank. The Swine/Texas isolate had only a partial sequence published on Genbank, but since amino acid residues along the entire length of the gene were of importance to this study, the PB2 gene was sequenced in its entirety. RNA extraction of virus stock culture was performed as described previously, and PB2

genes were amplified from extracted RNA by Reverse-Transcriptase Polymerase Chain Reaction using a Qiagen One-Step RT-PCR kit (Qiagen, Cat No. 210212). Two RT-PCR reactions were used to amplify the gene, resulting in two overlapping fragments. The first reaction generated a fragment of PB2 from nucleotide 1 to nucleotide 1341, and the other generated a fragment from nucleotide 1013 – 2341. Reactions were carried out under the following conditions on a GeneAmp 9700 Thermocycler:

Reagent	**Volume (µl)**	**Cycle Parameters**						
RNase-free H_2O	31	Stage 1 1X		Stage 2 35X			Stage 3 1X	
5X OneStep RT-PCR Buffer	10	48°C	95° C	94° C	51°C	72°C	72 °C	4 °C
dNTPs (10 mM)	2	30m	15m	1m	1m	1.5m	10m	Inf
Forward Primer (20 µm)	1							
Reverse Primer (20 µm)	1	Primers used were generated by Invitrogen and provided by collaborating investigator Dr. Erica Spackman but are unpublished as of yet and therefore not provided.						
Anti-RNase (20U/µl) (Ambion)	1							
OneStep enzyme mix	2							
Template RNA	2							
Final Volume	50							

PCR products were run on a 1% agarose gel to confirm the presence of the desired PCR product, and compared to a 1kb Plus molecular weight ladder (Invitrogen, Cat No. 10787-018) to determine the size. PCR reactions that yielded products of expected size were then cleaned using a Wizard PCR cleanup kit (Promega, Cat No. A9282). The DNA concentration was then determined using a Nanodrop, and 20 ng of the cleaned PCR product was sequenced using the same primers used for RT-PCR reactions.

Master mix composition for the sequencing reaction and PCR parameters were as follows:

Reagent	Volume (µl)
Nuclease-free H_2O	13.5
5X SEQ Buffer	3.5
BDT Ready Rxn Mix	1
Primer (20 µm)	1
Template (PCR product)	1
Final Volume	20

Cycle Parameters				
Stage 1 1X	Stage 2 25X			Stage 3 1X
96	96°C	45°C	60°C	4°C
2 min	1m	1m	1.5m	Inf

Primers used were supplied by Invitrogen and provided by collaborating investigator Dr. Erica Spackman but are unpublished as of yet, therefore their sequences were not provided.

Following PCR, the sequencing reactions were cleaned as follows: reactions were washed with a mixture of 2 µl of 125 mM EDTA plus 2 µl 3M Sodium Acetate plus 50 µl of 100% EtOH and incubated 15 minutes in the dark. Tubes were spun at 3000 x g for 30 minutes at 4 °C. Liquid was aspirated, the pellet was washed with 70% EtOH, and spun at 1650 x g for 15 minuts at 4 °C. EtOH was aspirated and any remainder was dried off at 95 °C. Twenty five microlitres of Hidi formamide was added, tubes were heated at 95 °C for 2 minutes, and then reactions were loaded onto the optical plate. Sequencing reactions were performed using BigDye Terminator kit (Applied Biosystems, Product No. 4337456) and run on an ABI Prism automated sequencer (ABI 3730, Applied Biosystems), and contigs were assembled using SeqMan 7.0 software (DNAStar).

14. Enzyme-Linked Immunosorbent Assays (ELISA) to Quantitate Cytokine Production

Supernatants from all time-course experiments were assayed for the production of TNF-α (R&D Systems, Cat No. PTA00), IL-1β (R&D Systems, Cat No. PLB100) and IFN-α (PBL Interferon Source, Cat No. 41100-1) using quantitative ELISA cytokine kits.

Frozen supernatants harvested at 24 and 48 hour time points were thawed and spun at 3000 RPM for 15 minutes prior to use. Only samples from plates incubated at 37°C were included for analysis. Procedures were performed as per manufacturer's instructions, except that standards, controls, and samples were added to the coated microplate strips and left overnight at 4°C rather than for 2 hours at room temperature. The optical densities of developed reactions were read at 450 nm, and cytokine concentrations in experimental samples were determined after extrapolating from the standard curve. In the event that supernatant from an uninfected control of a given experiment had a positive TNF-α reading, that value was subtracted from the TNF-α readings of the infected samples to correct for any factors other than infection with the influenza virus that contributed to TNF-α induction in the IPAMs.

15. Transformation of competent bacterial cells

XL10-Gold Ultracompetent Cells (Stratagene, Cat No. #200315) were transformed with plasmid DNA according to manufacturer's protocol with 12 different plasmids for the rescue of a recombinant influenza virus using reverse genetics. Plasmids used included six vRNA expression plasmids encoding genes from the SouthCarolina/1918 virus (pPol I-1918M, 1918NP, 1918NS, 1918PB1, 1918PB2, 1918PA), four protein expression plasmids encoding genes to create a functional polymerase complex (pCAGGS-NP, PA, PB1, PB2), and two vRNA/protein expression plasmids encoding the external glycoproteins of the Swine/Iowa virus (pHW2000/pDZ-1930HA, 1930NA). Plasmid maps of the pCAGGS and pPol I vectors are found in figures 2 and 3, respectively. Information on the pHW2000 vector is found in a

publication by Hoffmann *et al.* (2002). Briefly, 100 µl of ultracompetent cells were added to pre-chilled 14-ml polystyrene round-bottom tubes. Four microlitres of XL10-Gold beta-Mercaptoethanol was added to each tube, which were gently mixed prior to adding 1.0 µl of plasmid DNA (previously diluted to 20-50 ng/µl). A pUC18 control plasmid (Stratagene) and a non-transformed cell control were included with each set of transformations. After adding DNA, tubes were gently swirled and left on ice for 30 minutes. Cells were then heat-shocked for 45 seconds at 42°C, returned to ice for 2 minutes, then combined with 900 µl of pre-heated S.O.C. Medium (Invitrogen, Cat No. 46-0700) and incubated for 1hour at 37°C with shaking at 250RPM.

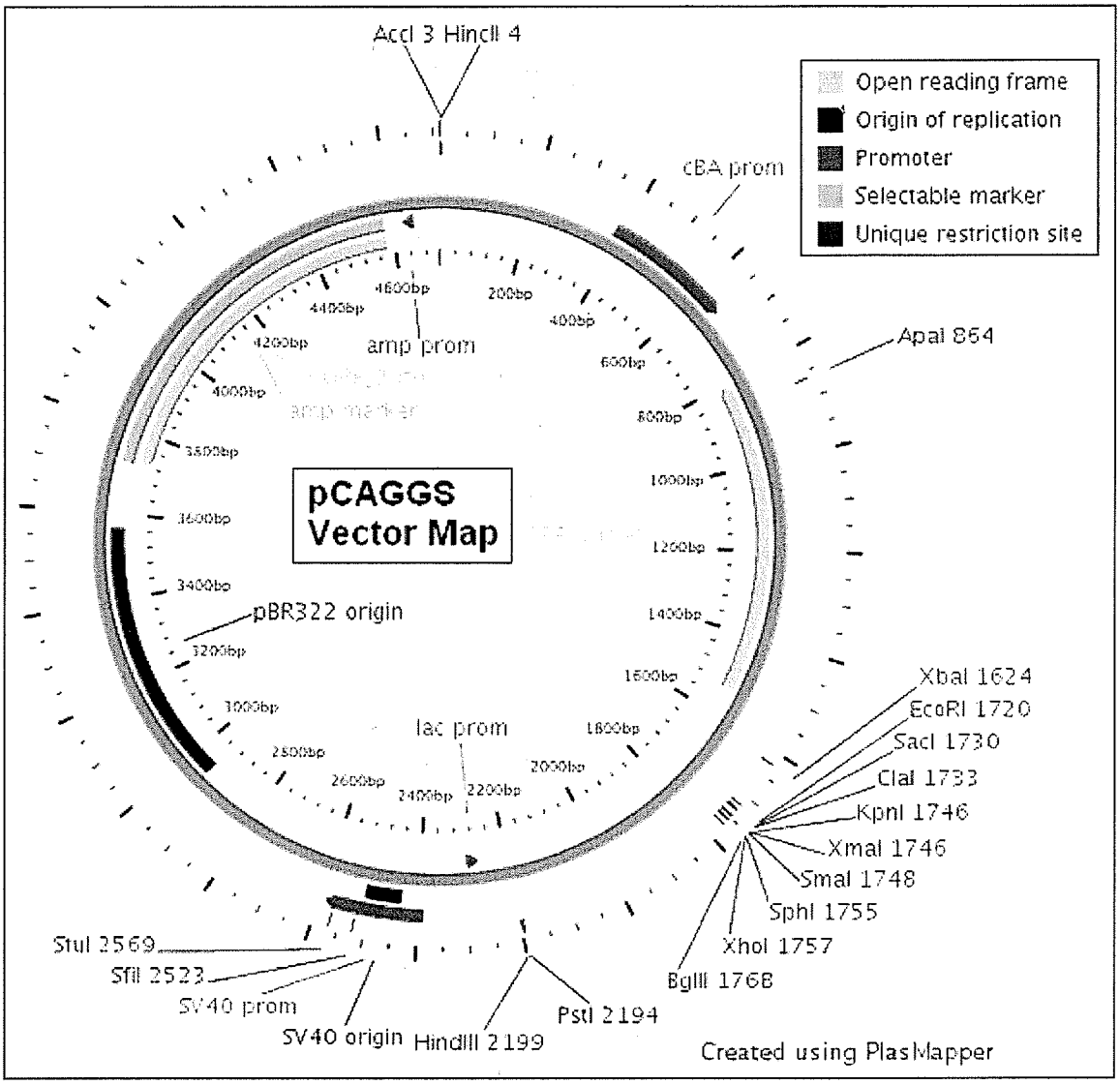


Figure 2. Map of pCAGGS vector used for protein expression plasmids (pCAGGS-PB1, PB2, PA, NP). The map was generated by entering the 4746bp sequence onto the PlasMapper (Version 2.0) server accessed online through http://wishart.biology.ualberta.ca/PlasMapper/ (Dong *et al.*, 2004). Note that only unique restriction sites are shown.

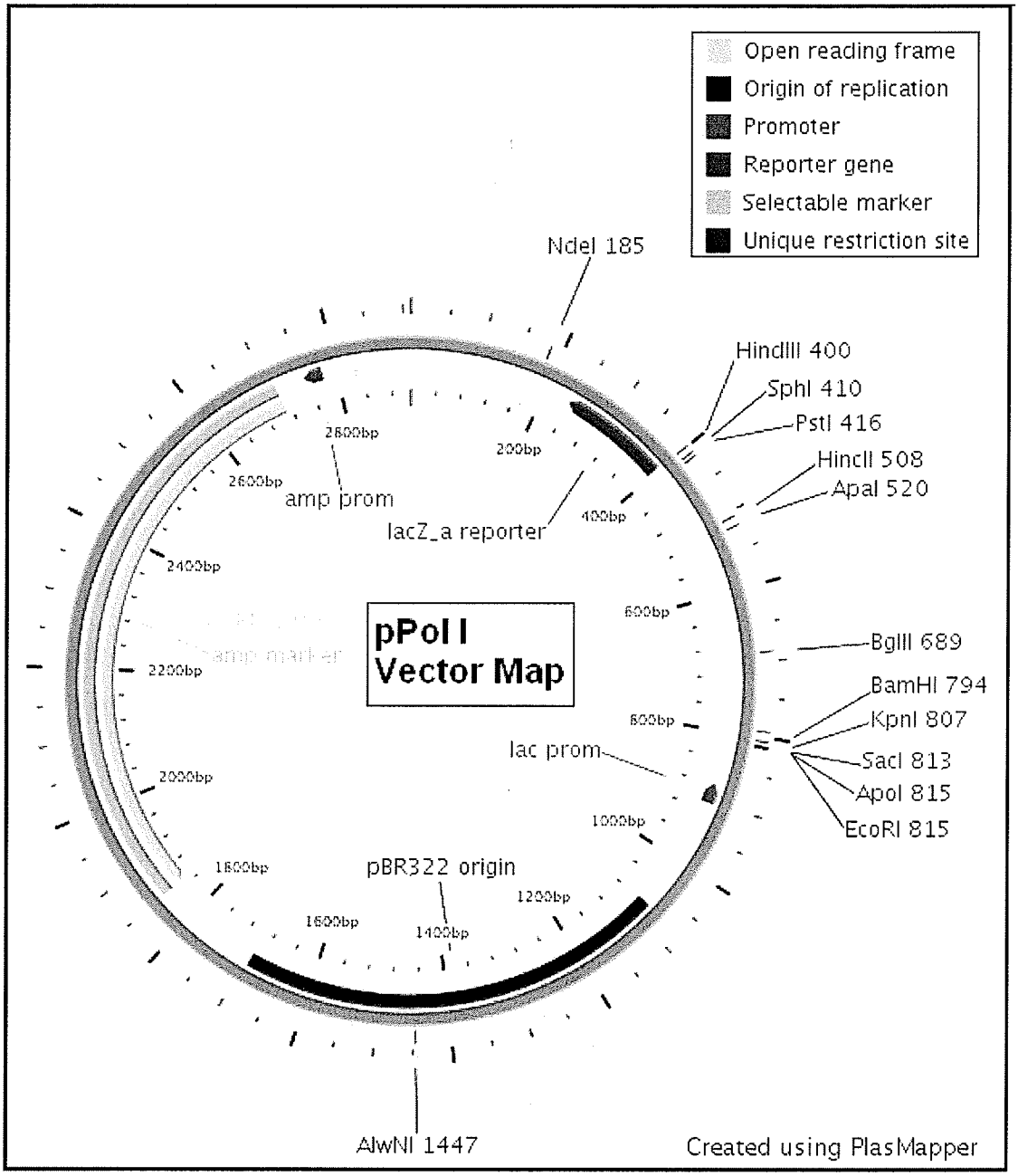


Figure 3. Map of pPol I vector used for SouthCarolina/1918 RNA expression plasmids. The map was generated by entering the 2911bp sequence onto the PlasMapper (Version 2.0) server accessed online through http://wishart.biology.ualberta.ca/PlasMapper/ (Dong *et al.*, 2004). Note that only unique restriction sites are indicated.

Approximately 100 µl of each transformation mixture was plated onto Luria Bertani (LB) Agar (Sigma, Cat No.L2897) plates containing 100 µg/ml carbenicillin (10 mg/ml in H2O), 12.5 µg/ml tetracycline (12.5 mg/ml in 50% EtOH), and 34 µg/ml chloramphenicol (34 mg/ml in 70% EtOH), and incubated overnight at 37°C. The following day, discrete colonies from each transformation were picked and used to inoculate 4 ml of LB broth (Sigma, Cat No. L3022) containing the same antibiotic concentrations as the LB agar plates. Tubes were incubated overnight at 37°C with shaking at 250-275 RPM, and cultures were analyzed the following day to confirm the presence of the plasmid insert.

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Cat No. 27106) in accordance with manufacturer's instructions. A 1.5 ml sample from each overnight culture was added to a 1.5 ml microcentrifuge tube (Diamed) and spun at 17,900 x g for 5 minutes (Eppendorf Centrifuge 5417R). Supernatants were discarded and the bacterial pellet was resuspended in 250 µl Buffer P1. Two hundred and fifty microlitres of Lysis Buffer P2 was then added and tubes were inverted five times and incubated at room temperature for five minutes. Three hundred and fifty microlitres of Neutralization Buffer N3 was then added, tubes were shaken vigorously five to ten times, and then spun at 17,900 x g for 10 minutes. Supernatants were then transferred to a Qiagen spin column and centrifuged for one minute at 17,900 x g. Flow-through was discarded after spinning and columns were washed with 500 µl of Binding Buffer PB and spun for 17,900 x G for one minute. Columns were then washed with 750 µl PE and spun for one minute at the same speed. After discarding flow-through, columns were spun once more at 17,900 x g for one minute to remove any residual wash buffer.

Columns were then placed into a clean 1.5 ml sterile microfuge tube and DNA was eluted with 50 µl buffer EB. Columns were left standing for one minute at room temperature, and then spun for one minute at 17,900 x g. DNA concentration of plasmid preps was determined by analyzing 2 µl of eluted DNA on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). The remaining eluted DNA was stored at 4°C.

16. Restriction Enzyme Analysis

To confirm the presence of the desired insert in the overnight culture of transformed cells, 0.5 - 1.0 µg of plasmid DNA was digested in a total reaction volume of 20 µl with the appropriate restriction enzyme(s) and buffer plus nuclease-free water (Ambion, Product No.AM9938) for a minimum of one hour at the appropriate temperature. For analysis of the 1918 pPolI plasmids, plasmid DNA was digested with 1.0 µl of XBA I (10 U/µl) (Roche, Cat No. 10674257001) in 2.0 µl SuRE/Cut Buffer H (10X Conc) (Roche, Cat No. 11417991001) at 37°C. With this vector XbaI cuts at positions 423 and 788 (not indicated on map), resulting in fragments with an excess of 350 bps. The pCAGGS helper plasmids were digested with 1.0 µl of XBA I and 1.0 µl of Xho I (40 U/µl) (Roche, Cat No. 10899194001) in 2.0 µl SuRE/Cut Buffer B (10X conc) (Roche, Cat No. 11417967001) at 37°C. The 1930 pDZ plasmids were digested with 1.0 µl of Apa I (10 U/µl) (Roche, Cat No. 10703745001) in 2 µl SuRE/Cut Buffer A (Roche, Cat No. 11417959001) at 30°C. Digested plasmids were run on a 1% agarose gel (Invitrogen, Cat No. 15510-027) and banding patterns were compared to a 1 Kb Plus DNA ladder (Invitrogen, Cat No. 10787-018) to check for the desired insert.

17. Sequencing of SouthCarlolina/1918 and Swine/Iowa Rescue Plasmids

Following restriction enzyme analysis to confirm the presence of the correct-sized insert, all rescue plasmids were sequenced by Katherine Handel and Tamiko Hisanaga at the NCFAD using an ABI Prism automated sequencer to confirm the identity of each gene and ensure no mutations were introduced following transformation. A complete list of sequencing primers used is found in Table 3.

18. Reverse Genetics – Virus Rescues

Attempts to rescue a recombinant virus bearing the external glycoproteins of the Swine/Iowa virus with the backbone of the SouthCarolina/1918 influenza virus were carried out using the method described by Fodor *et al.* (1999) with modifications. 293T cells were transfected with eight vRNA transcription plasmids and four protein expression plasmids to generate infectious virus, and supernatants were then passaged onto MDCKs to amplify the rescued virus. The day prior to transfection 293T cells were seeded on 6-well plates at a density of approximately 50,000 cells/cm^2, resulting in 50-70% confluence by the next day. On the day of transfection, each reaction was set up in individual 1.5 ml sterile tubes. Two hundred microlitres of OPTI-MEM (Gibco, Cat No. 51985034) was added to each tube followed by 10 µl of TransIT-LT1 transfection reagent (Mirus, Cat No. MIR 2300) or 10 µl of Lipofectamine^{TM} 2000 (Invitrogen, Cat No. 11668-027) (warmed to room temperature). Tubes were gently stirred and left to incubate at room temperature for 5-10 minutes. A transfection mixture was made in a separate tube and consisted of 0.1 µg of the 8 vRNA transcription plasmids (pPolI-1918PB2, 1918PB1, 1918PA, 1918M, 1918NP, 1918NS, pDz-1930HA and pDz-

Table 3. Sequencing primers used for SouthCarolina/1918 and Swine Iowa gene segments.

Primer Name	Primer Specificity	Primer Sequence
3' PB1 NCR	Influenza virus	AGCGAAAGCAGGCAAACCATTTGAATGG
3' PB2 NCR	Influenza virus	AGCGAAAGCAGGTCAATTATATTCAATATGG
3' PA NCR	Influenza virus	AGCGAAAGCAGGTACTGATTCGAAATGG
3' NP NCR	Influenza virus	AGCAAAAGCAGGGTAGATAATCACTCACTGAGTG
3' M NCR	Influenza virus	AGCAAAAGCAGGTAGATATTGAAAGATG
3' NS NCR	Influenza virus	AGCAAAAGCAGGGTGGCAAAGACATAATGG
3' HA NCR	Influenza virus	AGCAAAAGCAGGGGAAAATAAAAACAACC
3' SW30 NA NCR	1930 Influenza virus	AGCGAAAGCAGGGGTTTAAAATGAATAC
5' PB1 NCR	Influenza virus	AGTAGAAACAAGGCATTTTTTCACGAAGGACAAGCC
5' PB2 NCR	Influenza virus	AGTAGAAACAAGGTCGTTTTTAAATTATTCG
5' PA NCR	Influenza virus	AGTAGAAACAAGGTACTTTTTTGGACAGTATGG
5' NP NCR	Influenza virus	AGTAGAAACAAGGGTATTTTTCATTAATTGTCG
5' M NCR	Influenza virus	AGTAGAAACAAGGTAGTTTTTTACTCTAGC
5' NS NCR	Influenza virus	AGTAGAAACAAGGGTGTTTTTTATCATTAAATAAGC
5' HA NCR	Influenza virus	AGTAGAAACAAGGGTGTTTTTTCTTATATTTCTGAA ATTCTGG
5' NA NCR	Influenza virus	AGTAGAAACAAGGAGTTTTTTTTCAACGG
PB1 401F	1918 Influenza virus	ATAGGAACCAGCCTGCTGCAACAGCATTGGCC
PB1 874F	1918 Influenza virus	AATTCTCAGGACACTGAGCTTTCTTTCACC
PB1 1327F	1918 Influenza virus	TCTTCTGATGATTTTGCTCTGATTGTGAATGCACCC
PB1 1801F	1918 Influenza virus	ATTAGAAATCTCCACATTCCTGAAGTCTGC
PB2 361F	1918 Influenza virus	AAAGTCGAAAGGTTAAAACATGGAACCTTTGGCCC
PB2 822F	1918 Influenza virus	AGATTCACTAGCATCTCTGTTGGAAATGTGCCACAGC
PB2 1250F	1918 Influenza virus	ATCTGAATTTCGTTAATAGGGCGAATCAGCG
PB2 1642F	1918 Influenza virus	AACACCTATCAGTGGATCATCAGAAACTGGG
PA 441F	1918 Influenza virus	TTTCTCGTTCACTGGGGAAGAAATGGCC
PA 874F	1918 Influenza virus	ATTGAGGACACAAGCCATGAAGGAGAGGGG
PA 1335F	1918 Influenza virus	TTTCACAGCAGAGGTGTCTCATTGCAGAGCC
NP 395F	1918 Influenza virus	TTGTCTGACTCACATGATGATCTGGC
NP 809F	1918 Influenza virus	TTGCTCACAAGTCCTGCCTGCCTGCC
HA 395F	1918 Influenza virus	AAATATTTCCCAAGACAAGCTCGTGGCCC
HA 902F	1918 Influenza virus	ATT GTG CTA TAA ACA GCA GTC TCC CTT TCC
HA 784R	1918 Influenza virus	TTG CCT CAA ATG TTA TTG TGT CTC CGG G
NA 435F	1918 Influenza virus	AAA TGG GAC CGT CAA GGA CAG AAG CCC C
NA 933F	1918 Influenza virus	TTA TCA AAT AGG GTA CAT CTG CAG TGG GG
PpolIFor	pPolI plasmid	TTTTTGGGGACAGGTGTCCGTG
PpolIRev	pPolI plasmid	AGTCCCATTCGCCATTACCG

1930NA), and 1.0 μg of each protein expression plasmid (PCAGGS-PB2, PB1, PA, NP). Rescue plasmids for a control virus, A/PR/8/34, were also used to transfect the 293T cells, as this virus was successfully rescued previously. Rescue plasmids for the PR/8 virus, which include modified pPol I RNA expression plasmids (Appendix III) and the pCAGGS helper plasmids, were provided by Dr. Darwyn Kobasa at the National Microbiology Laboratory in Winnipeg. The mixture was added to the reaction tubes and incubated for 30 minutes at room temperature.

While the plasmid mixture was incubating, the plate of 293T cells was washed gently with 2.0 ml of pre-warmed OPTI-MEM per well. One millilitre of OPTI-MEM was then added to each well, and cells were incubated at 37°C until required. After 30 minutes of incubation, the transfection reaction mixture was added drop-wise to each well and plates were gently rocked to disperse the solution. Each transfection condition was performed in duplicate wells, and two control wells of plasmid-free transfection mixtures were also included. Transfected cells were incubated at 37°C for 48 hours.

Following two days of incubation, cells were checked for CPE and supernatants were harvested into individual tubes. Three microlitres of TPCK-trypsin (1.0 mg/ml concentration) were added to each tube of harvested supernatant, gently mixed and incubated for approximately 15-30 minutes at 37°C. During incubation, MDCK cells of approximately 70% confluency (seeded one day prior on 12-well plates) were washed twice with 1.0 ml of pre-warmed MEM-BSA (AMEM with a final concentration of 0.3.% BSA and 1.0 μg/ml TPCK-Trypsin). After washing 2ml of MEM-BSA was added to each well, along with varying amounts of TPCK-Trypsin-activated transfection supernatant (ranging from 1.0 μl to 100 μl per well). MDCKs were monitored for signs of infection,

and supernatants from wells demonstrating CPE were harvested and stored at –80°C to further amplify the resulting virus.

Results{tc "Results"}

1. Characterization of IPAMs by Flow Cytometry and Confocal Microscopy{tc "Characterization of IPAMs by Flow Cytometry and Confocal Microscopy"}

To determine whether both avian- and mammalian-tropic influenza A viruses would recognize receptor molecules on IPAMs, the cells were visualized with a confocal microscope for the presence of α-2,3 and α-2,6 sialic acid linkages. Both α-2,3 and α-2,6 sialic acid residues were found present on the surface of IPAMS in roughly equal proportions as determined by co-localization of red and green fluorescence signals on the surface of each cell (Figure 4). Lectin staining and confocal microscopy performed by collaborating investigators showed duck tracheal sections were positive for α-2,3-linked receptors (using SNA) while negative for α-2,6-linked receptors (MAA), confirming the specificity of these agents (data not shown).

The monomyeloid nature of IPAMs was previously determined upon establishment of the cell line (Weingartl *et al.*, 2001). However, cell maintenance media used in this project differed from that in published work, so it was necessary to confirm expression of monocyte-specific surface markers. Flow cytometry results showed that IPAMs were SWC3+, had very low level of CD14 expression, and no expression of SWC9 (Figure 5).

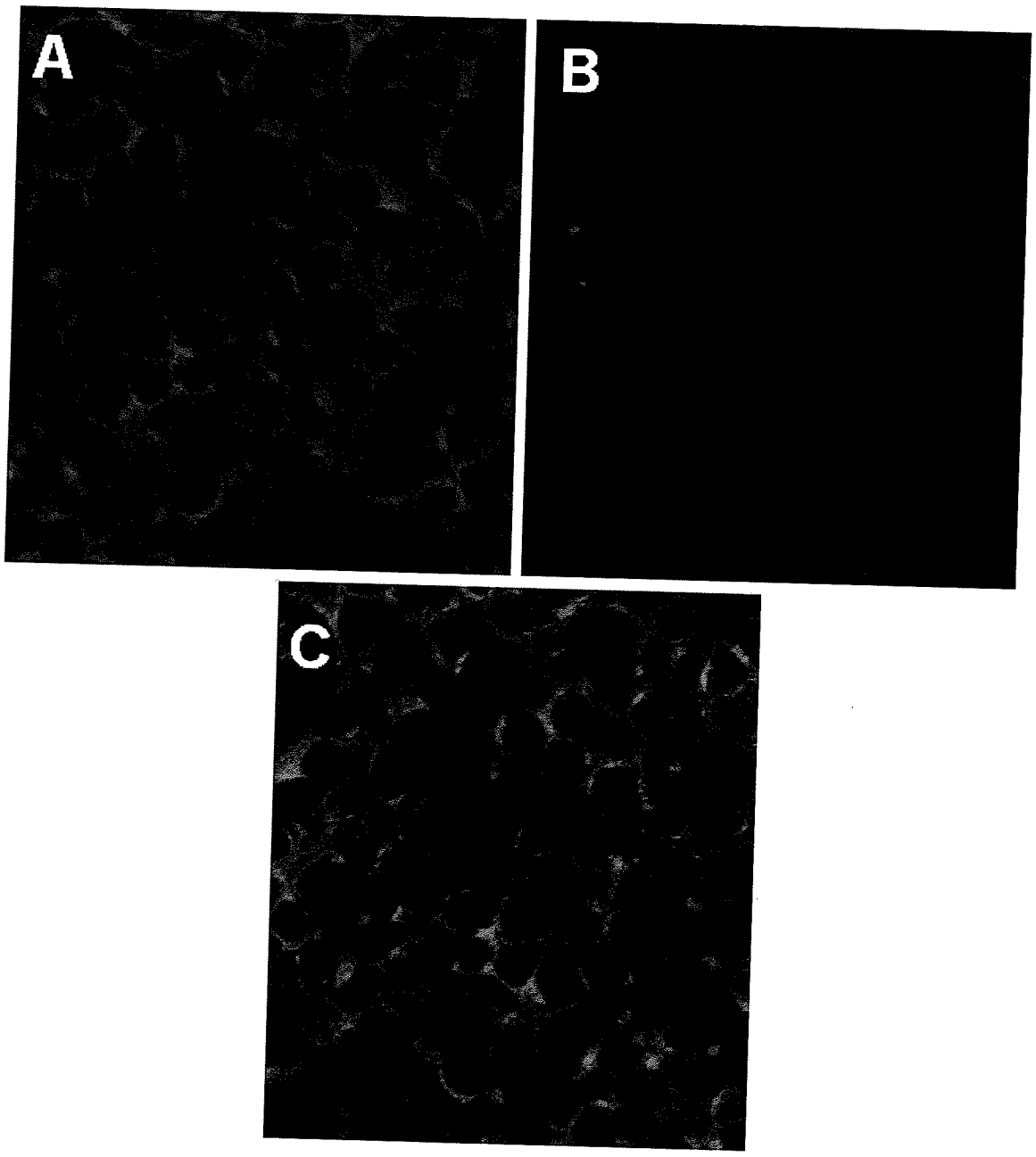


Figure 4. Localization of alpha-2,3 and alpha-2,6-linked sialic acid species on the surface of IPAMs (Clone 3D4/31). A) Cells were single-stained with SNA (green) to detect alpha-2,3 linkages or B) with MAA (red) to detect alpha-2,6-linkages. C) Double-stained cells with SNA and MAA to determine co-localization of alpha-2,3 and alpha-2,6 linkages (yellow). Cells were visualized on an Olympus Fluoview FV300/500/1000c confocal microscope.

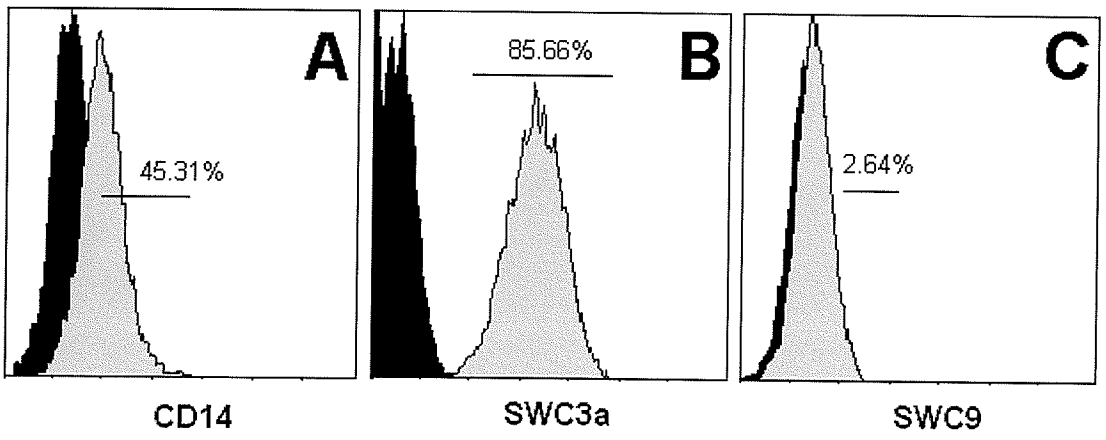


Figure 5. Surface expression of monocyte and macrophage-specific markers on uninfected IPAMs (clone 3D4/31) as determined by flow cytometric analysis on a Beckman-Coulter FC500 cytometer. Overlay plots represent unstained control cells (black) and IPAMs stained for detection of various markers (grey) A) CD14, B) Porcine pan-myeloid marker SWC3a, and C) Porcine macrophage marker SWC9.

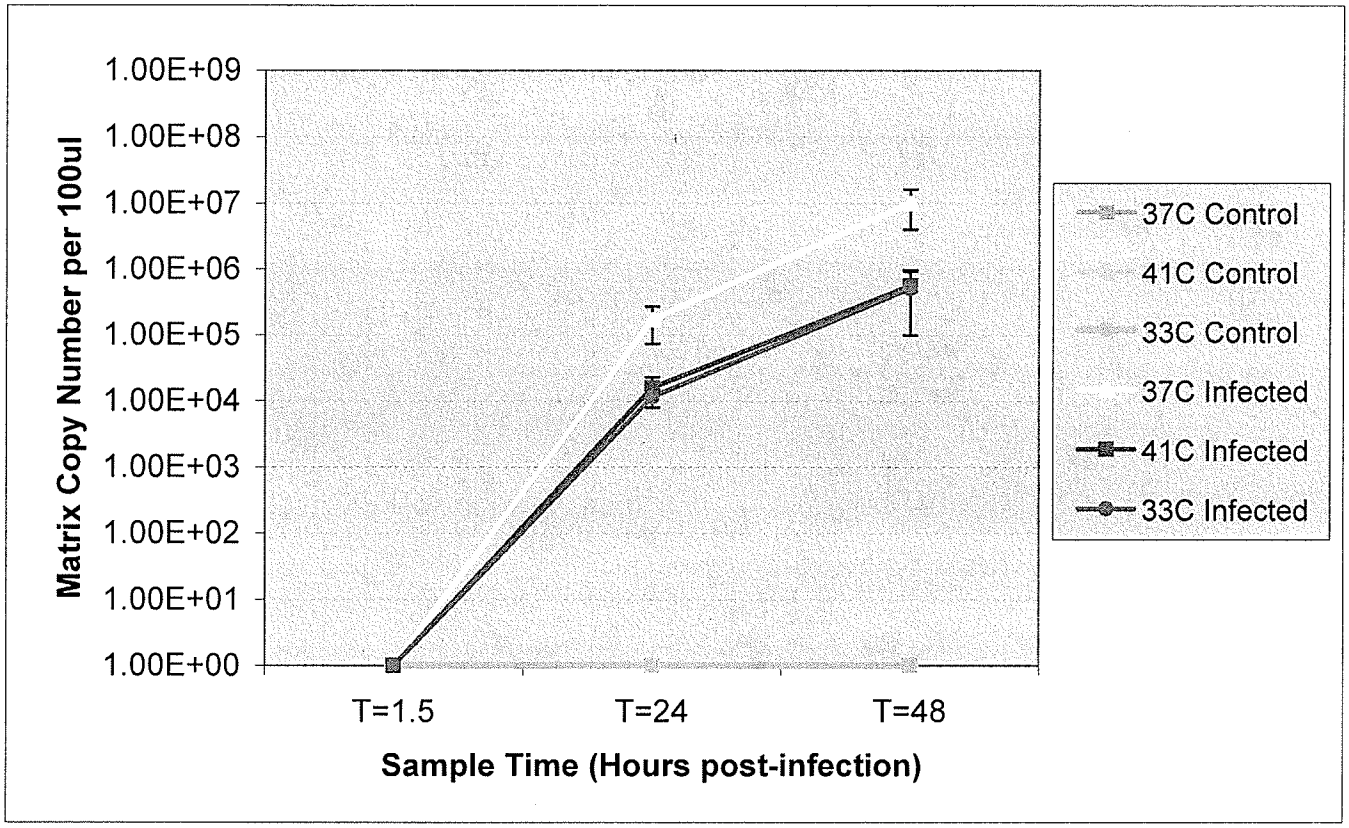
2. Replication of Influenza A Viruses in IPAMs at 33°C, 37°C and 41°C{tc "Replication of Influenza A Viruses in IPAMs at 33°C, 37°C and 41°C"}

2.1. Replication of Swine Viruses

The two swine viruses studied did not show a general preference for specific incubation temperatures. Following 24 hours of incubation at 33°C, 37°C, and 41°C, the Swine/Iowa virus showed a strong preference for 37°C, reaching titres one log higher than those reached at the other two temperatures. This pattern remained consistent through 48-hours post-infection, where RNA levels at 37°C were significantly higher than those at 33°C or 41°C ($p<0.05$) while the difference between levels at 33°C and 41°C was minimal (Figure 6). Visual examination of infected cells further supported these results, with massive CPE observed at 37°C and only slight CPE at 33°C or 41°C following 48 hours of incubation (Table 4, Figure 7). HA values reflected CPE results, as supernatants from 37°C-incubated plates typically had HA values several-fold higher than 33°C and 41°C supernatants (Table 5). To confirm that these differences also correlated to actual titres of infectious virus, $TCID_{50}$ assays were conducted using supernatant harvested from Swine/Iowa-infected IPAMs at 37°C and 41°C. MDCK cells infected with supernatant from 37°C Swine/Iowa experiments had a mean titre of $2.25x10^7$ pfu/ml, whereas supernatant from 41°C experiments had a mean titre of only $4.42x10E^4$ pfu/ml, indicating that RT-PCR results were an accurate indication of virus titres.

The Swine/Texas virus showed a different temperature preference from the Swine/Iowa isolate. Like Swine/Iowa, this virus also reached its highest RNA titres at 37°C by 24-hours post-infection, but titres at 33°C were almost identical to those at 37°C

while RNA levels at 41°C were only half of those reached at the other two temperatures (Figure 8). Biologically, this difference was not as significant, as CPE was observed in all experimental conditions (Table 4). Hemagglutinating activity was also present in supernatants from all incubation temperatures, and average HA values were equal in the 37°C, 33°C, and 41°C samples. By 48 hours post-infection there was still a significant difference between titres at 33°C and 41°C ($p<0.05$) (Figure 8). This was again reflected in hemagglutination abilities of the infected supernatants, with 37°C and 33°C samples generally producing the same HA values of 16-32 HAU, while 41°C samples had slightly lower HA values of 8-16HAU (Table 5). When infected cells were visually examined after 48 hours under each of the three incubation conditions, massive CPE was noted in all cases (Table 4).



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.01	0.002
41	37	0.27	0.004
33	41	0.122	0.904

Figure 6. Growth properties of A/Swine/Iowa/15/1930 (H1N1) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.

Table 4. Cytopathic effect (CPE) of influenza viruses on IPAMs at 24 and 48-hours post-infection following incubation at 37°C, 33°C, or 41°C. CPE scoring was based on comparison of infected wells and control wells from the same plate of IPAMs incubated at each of the three experimental conditions.

Experimental Isolate	Cytopathic Effect					
	24 Hours Post-Infection			48 Hours Post-Infection		
	33C	37C	41C	33C	37C	41C
A/Duck/AB/C-16/2007	-	-	+	+	++	+++
A/Chicken/Vietnam/14/2005	N/a	N/a	N/a	N/a	N/a	N/a
A/Emu/Texas/39924/93	-	++	++	+	+++	+++
A/Chicken/BC/514/2004	-	+++	+++	++	+++	+++
A/Swine/Iowa/15/1930	-	+	-	+	+++	++
A/Swine/Texas/4199-2/98	++	++	++	++	+++	+++
A/SouthCarolina/1918	N/a	N/a	N/a	N/a	N/a	N/a
A/WS/33	-	-	-	++	++	-
A/PR/8/34	-	-	-	++	+++	++
A/HongKong/8/68	-	-	-	-	-	-

+++ = Severe CPE, ++ = Moderate CPE, + = Mild CPE, - = No CPE, N/A = No data available.

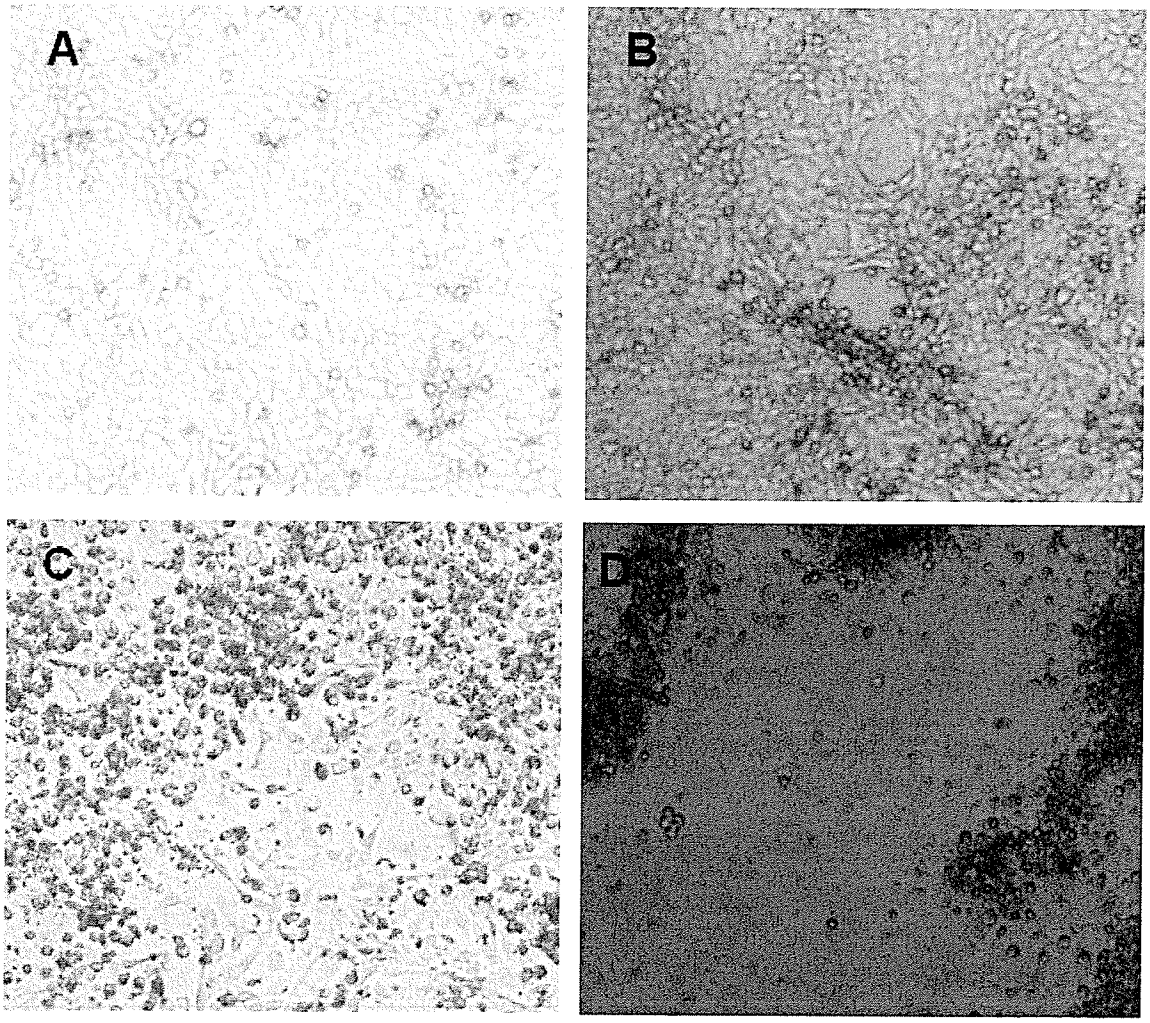
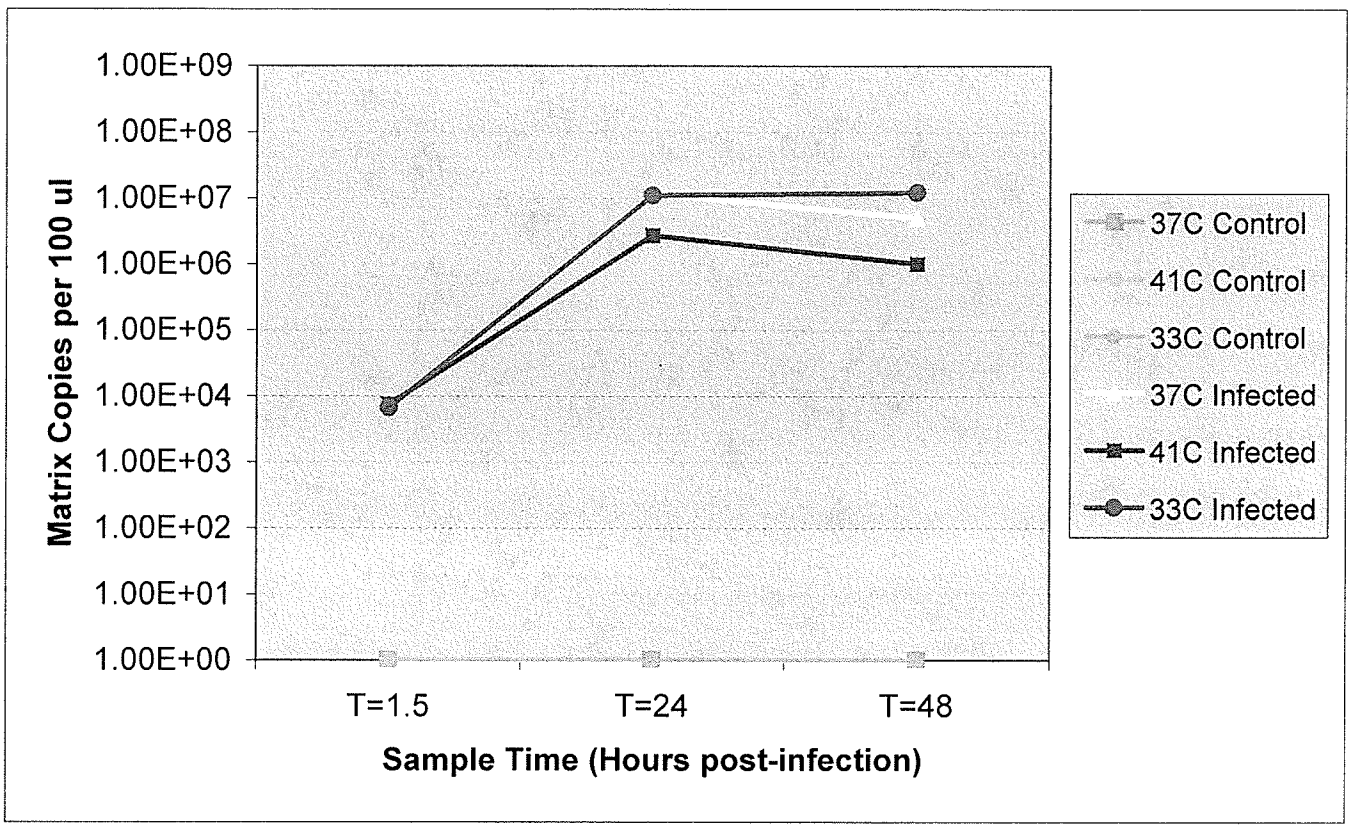


Figure 7. Representative examples of CPE following infection of IPAMs with influenza virus. CPE Scoring was based on comparison of infected wells and control wells from the same plate of IPAMs incubated at each of the three experimental conditions. A. No CPE, B. Mild CPE, C. Moderate CPE, D. Severe CPE.

Table 5. Hemagglutination assay titres of supernatants from influenza virus-infected IPAMs at 24 and 48-hours post-infection following incubation at 37°C, 33°C, or 41°C. Values reflect results from a single representative experiment at each temperature for all viruses.

Experimental Isolate	HA Titres					
	24 Hours Post-Infection			48 Hours Post-Infection		
	33C	37C	41C	33C	37C	41C
A/Duck/AB/C-16/2007	16	16-32	16-32	32-64	32-64	32-64
A/Chicken/Vietnam/14/2005	n/d[a]	8	8	n/d	8	8
A/Emu/Texas/39924/93	0	8	8	0	64	64
A/Chicken/BC/514/2004	4	32-64	32-64	64	64	64
A/Swine/Iowa/15/1930	0	8	0	8-16	256	8
A/Swine/Texas/4199-2/98	16	16	4	16-32	8-16	8
A/SouthCarolina/1918	0	0	0	0	0	0
A/WS/33	0	0	0	8	4	0
A/PR/8/34	0	8-16	0	128	128	8-16
A/HongKong/8/68	0	0	0	0	0	0

[a] n/d – No Data available.



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.989	0.603
41	37	0.158	0.093
33	41	0.188	0.027

Figure 8. Growth properties of A/Swine/Texas/4199-2/98 (H3N2) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of < 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.

2.2. Replication of Avian Viruses

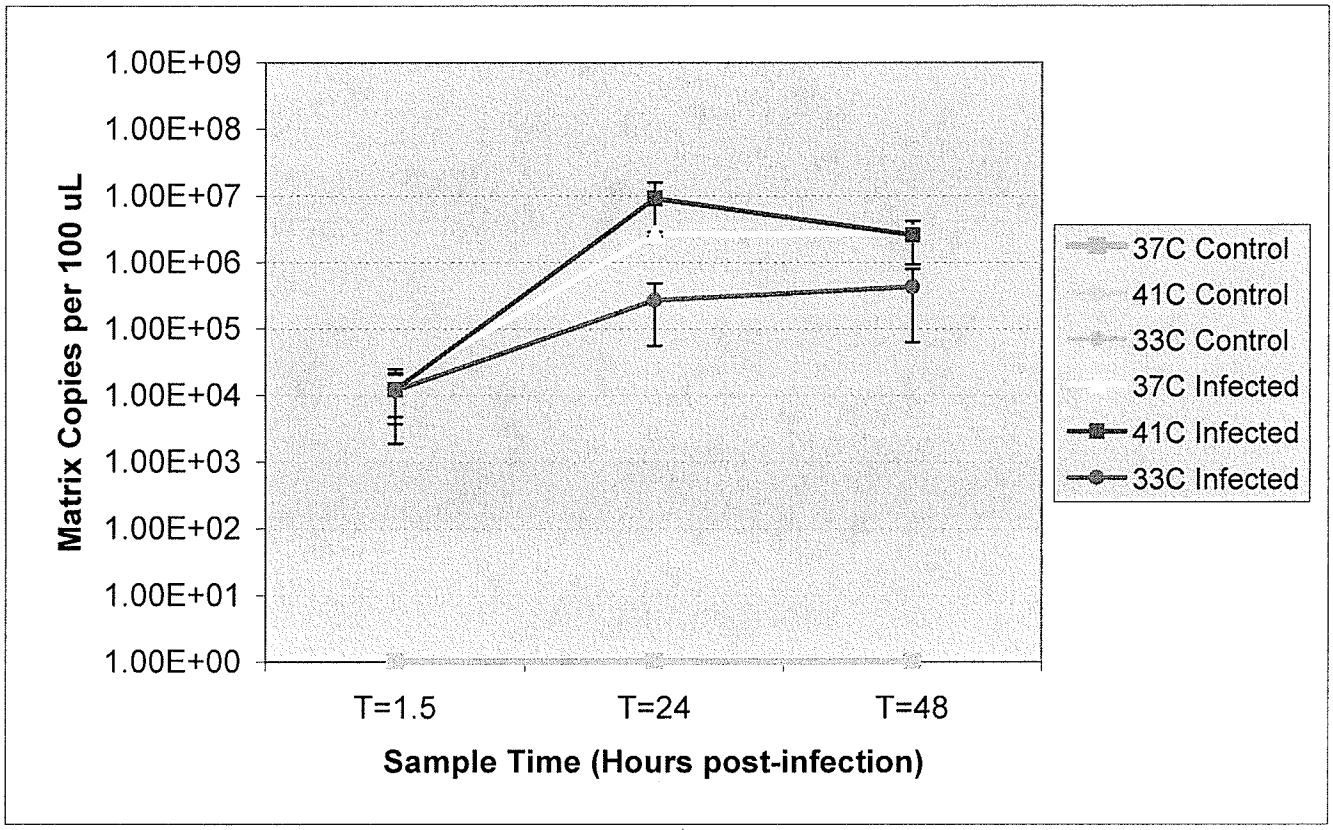
As a group, the avian influenza viruses generally preferred higher incubation temperatures (37°C and 41°C) to cooler conditions, and this was observed throughout the time-course and particularly true with regards to RNA levels. After 24 hours of growth at the three different experimental temperatures, the Duck/Alberta isolate reached significantly higher titres at 41°C and 37°C compared to 33°C ($p<0.05$). This was reflected by HA values and CPE observed in infected wells, as CPE was notable by 24 hours in cells incubated at 41°C and 37°C compared to control cells, but not at 33°C. By 48 hours post-infection, cells subjected to 41°C incubation showed very strong CPE, those kept at 37°C had less severe CPE and ones at 33°C were only beginning to show signs of infection (Table 4). RNA levels and HA values at 48-hours post-infection, however, did not show any statistically significant difference between 33°C and 37°C or 33°C and 41°C (Figure 9).

Like the Duck/Alberta isolate, the Chicken/Vietnam virus showed a preference for 41°C and 37°C over 33°C ($p<0.05$) after 24 hours of infection. The preference for higher temperatures was maintained through the 48-hour sampling time, at which point the RNA levels between 37°C and 33°C-incubated cells were still significantly different ($p<0.05$), though a significant difference between 33°C and 41°C was no longer observed due to a drop in RNA levels at 41°C (Figure 10).

The Emu/Texas virus also displayed the typical avian temperature preference, reaching titres nearly 2 logs higher at 37°C and 41°C than at 33°C after 24 hours of infection. By 48-hours post-infection the difference between RNA levels at 37°C and 33°C was still significant ($p<0.05$)(Figure 11). CPE observations and HA results

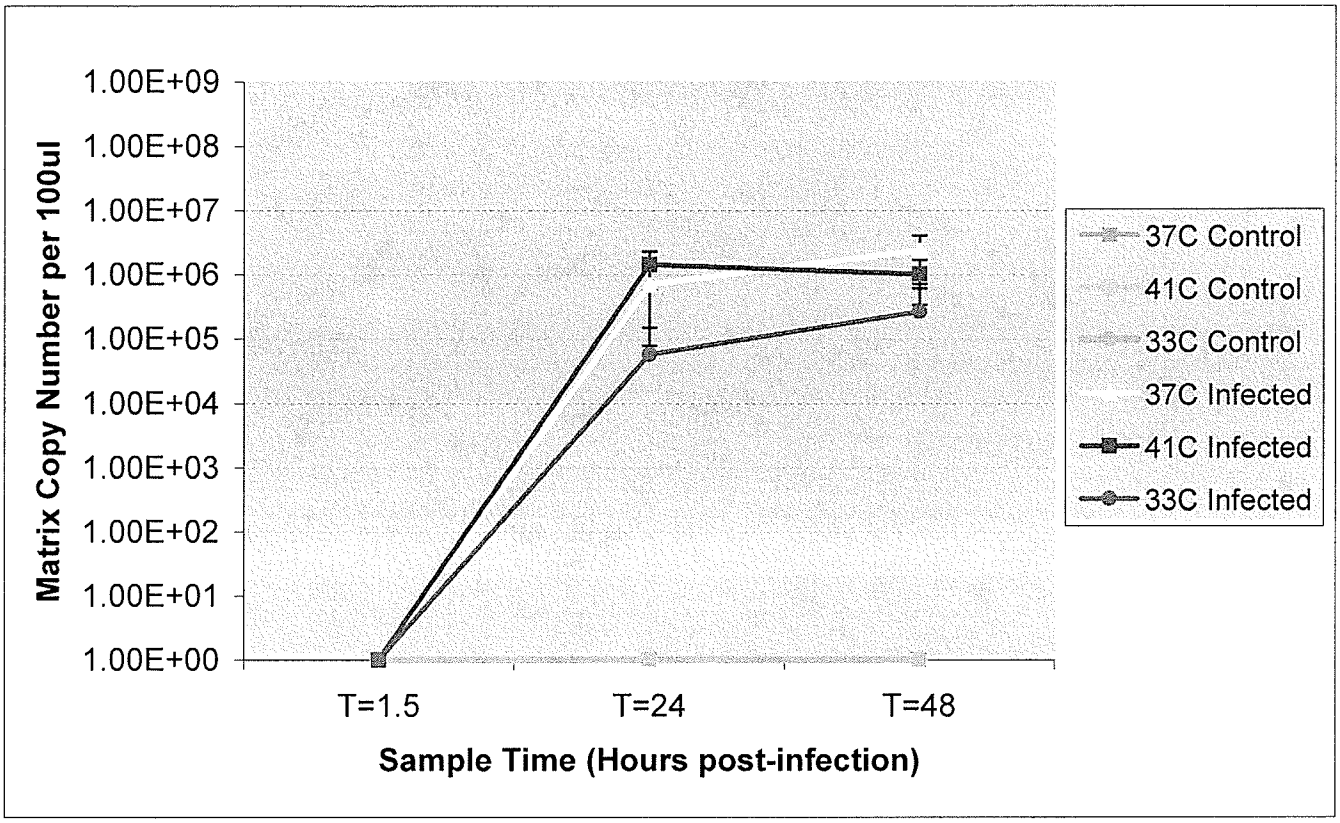
reaffirmed these differences; with HA values of 16-64 HAUs recorded for samples incubated at 37°C and 41°C while those from 33°C-incubated plates had no hemagglutination ability (Tables 4 and 5).

The final avian virus examined, CK/BC, behaved like the other viruses at 24 hours post-infection, with peak RNA levels found in 41°C incubated cells and lower amounts in 37°C followed by 33°C incubated plates. By 48 hours post-infection, however there were no statistically significant differences in RNA titres between any of the three temperatures (Figure 12). CPE scores did not entirely reflect this observation, as 33°C-infected plates did not show the same degree of CPE as the higher temperatures at 48 hours post-infection (Table 4). However, when HA values were compared from 48-hour samples, supernatants from 37°C-incubated plates generally had the same HA titres as supernatants from 33°C and 41°C-incubated plates by this time-point, ranging from 16 to 128 HAU (Table 5).



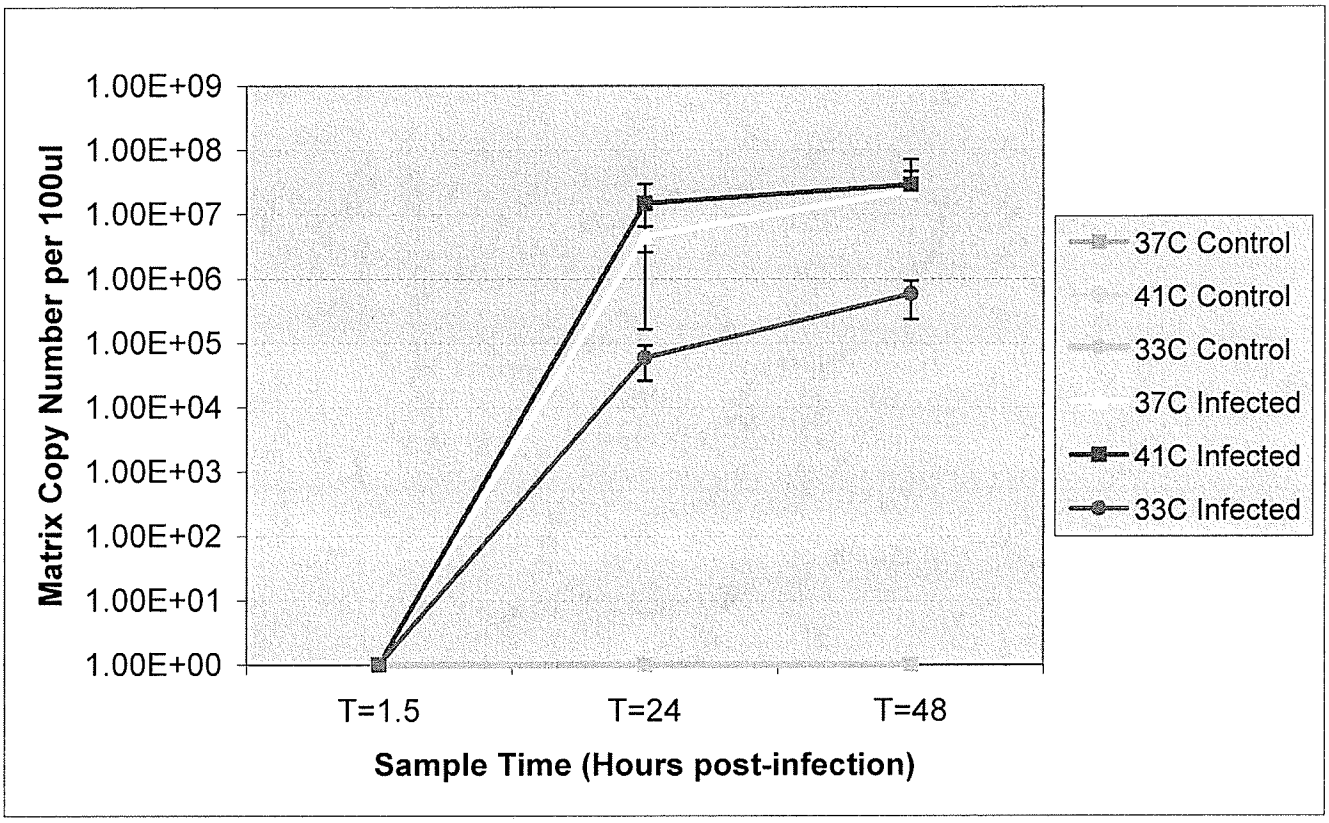
Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.018	0.078
41	37	0.34	1
33	41	0.004	0.08

Figure 9. Growth properties of A/Duck/Alberta/C-16/2007 (H7N7) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



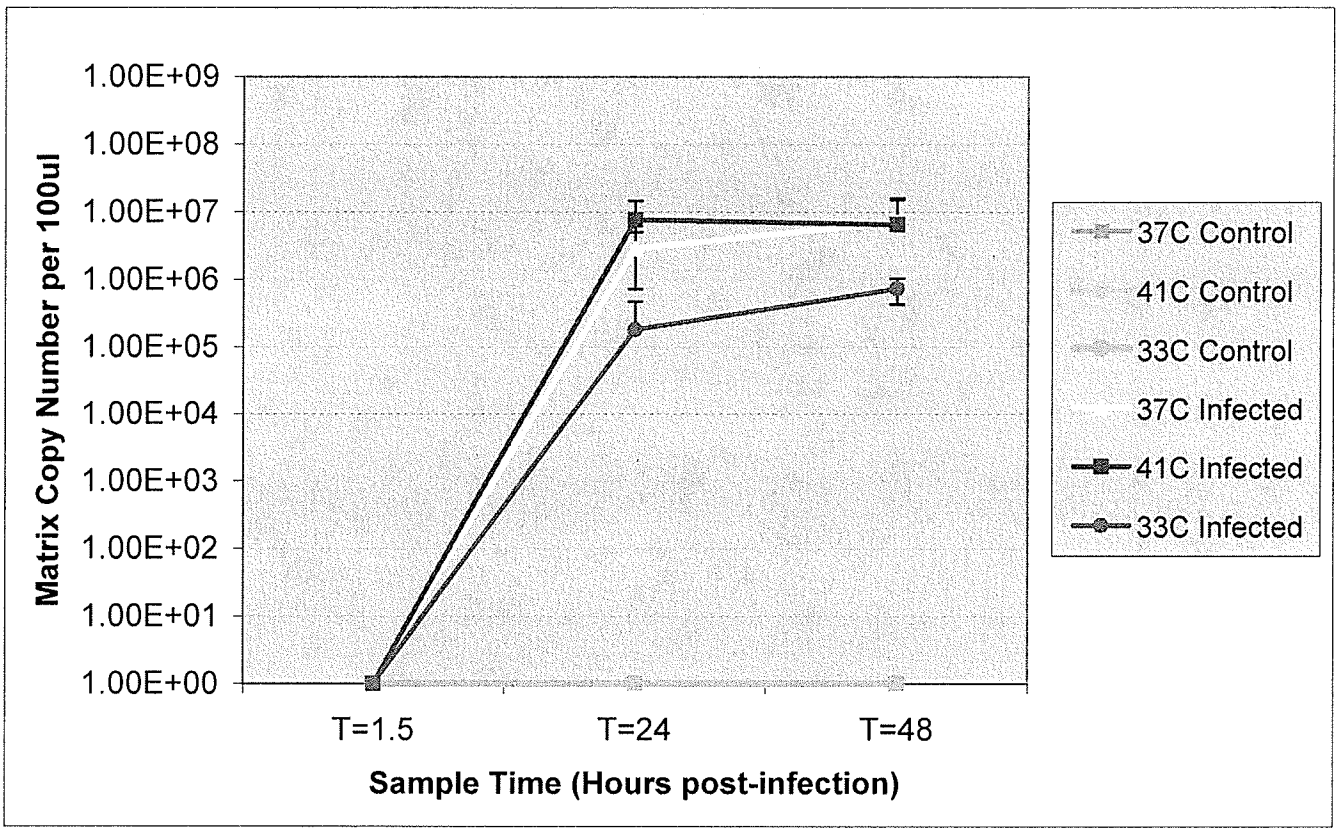
Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.01	0.012
41	37	0.801	0.533
33	41	0.009	0.133

Figure 10. Growth properties of A/Chicken/Vietnam/14/2005 (H5N1) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.0001	0.021
41	37	0.126	0.944
33	41	0.0001	0.065

Figure 11. Growth properties of A/Emu/Texas/39924/93 (H5N2) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.056	0.316
41	37	0.816	0.808
33	41	0.043	0.198

Figure 12. Growth properties of A/Chicken/BC/514/2004 (H7N3) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.

2.3. Replication of Human Viruses

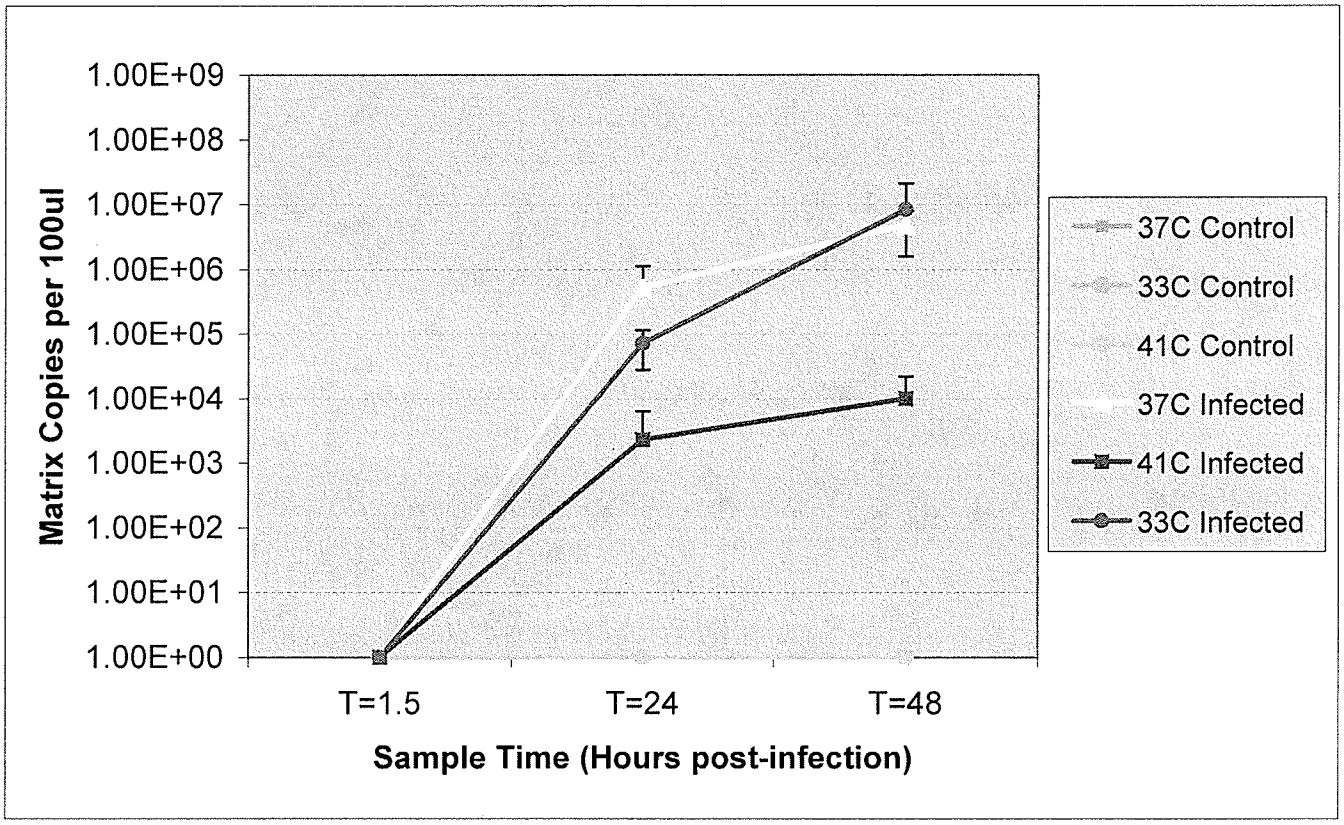
The comparative growth rates of four human influenza viruses showed a general preference for temperatures typical of a human host, though the SouthCarolina/1918 virus was an exception to this trend. WS/33, consistently showed a strong preference for temperatures associated with the human respiratory tract, reaching titres up to three logs higher at 37°C and 33°C than 41°C, and differences in RNA replication rates were significant between these temperatures at 24 and 48 hour times-points (Figure 13). This was also supported by CPE observations, as no CPE was documented in 41°C-incubated cells at any time post-infection (Table 4).

The PR/8 virus showed a different temperature preference from the WS/33 virus, replicating to very high titres at 37°C by 24 hours post-infection, but reaching significantly lower titres at 41°C and at 33°C ($p<0.05$) (Figure 14). This was evident in hemagglutinating ability of the different samples, as 37°C samples had relatively high HA values compared to a complete lack of hemagglutination in the 33°C and 41°C samples (Table 5). After 48 hours of infection, CPE was evident in all infected wells from plates at each of the three incubation conditions (Table 4), though HA values RNA levels were significantly lower in the 41°C-incubated samples compared to those at 37°C and 33 °C, indicating the continued preference for the temperatures typical of a human host (Figure 14) (Table 5).

Unlike both the WS/33 and PR/8 isolates, the HongKong/68 virus showed no significant differences in RNA levels, CPE or HA values at any of the three temperatures tested by 24 hours post-infection (Figure 15) (Table 4). By 48 hours post-infection, however, this virus exhibited a temperature preference similar to the WS isolate, showing

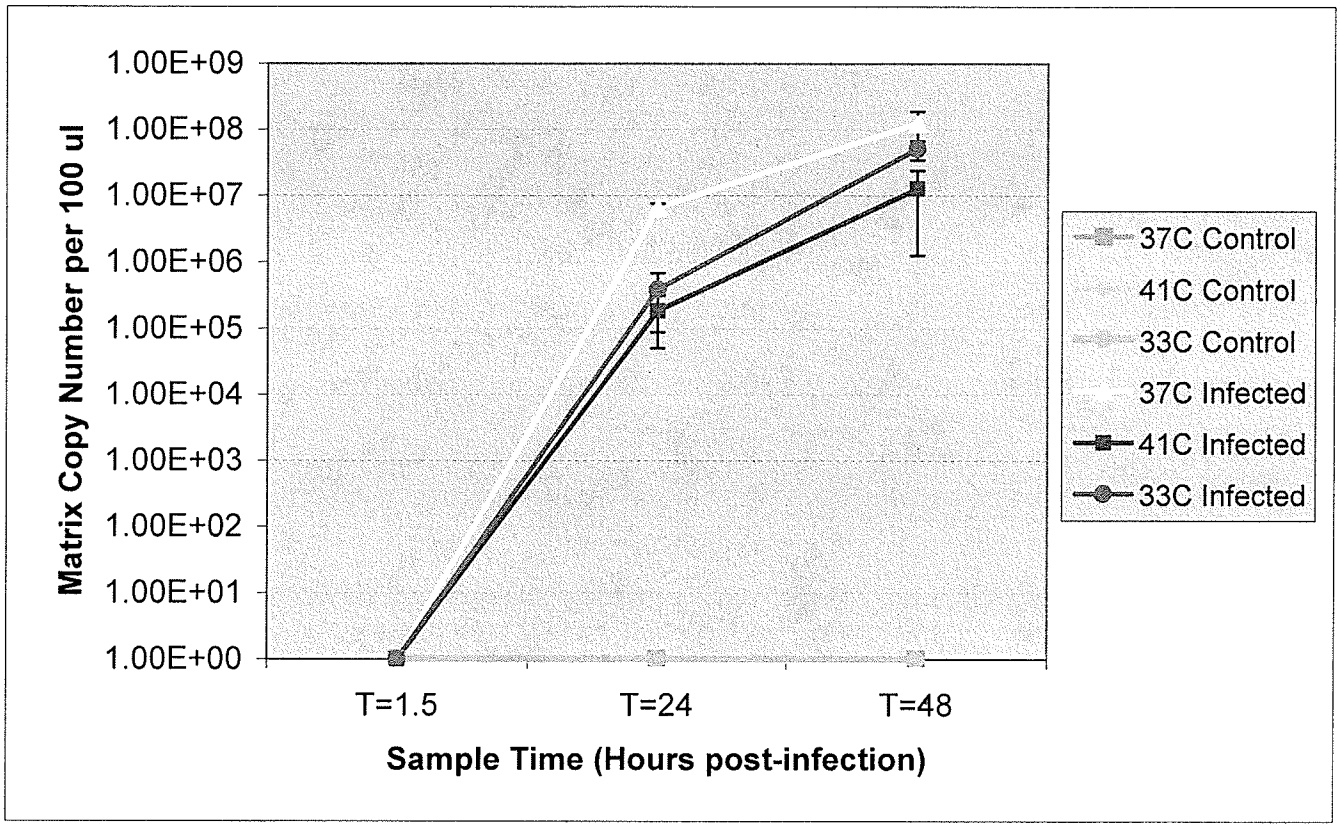
significant differences in RNA levels when 37°C and 41°C or 41°C and 33°C-incubated samples were compared ($p<0.01$) (Figure 15). This virus was also unusual because no hemagglutinating ability or discernable CPE was found in any of the infected samples at 24 or 48 hours post-infection (Table 4). To ensure that RNA levels did in fact correspond to infectious virus particles, infected and control supernatants from two independent HongKong/68-IPAM infections were passaged on confluent wells of MDCK. By 24 hours post-infection, MDCKs inoculated with infected IPAM supernatants from 33°C or 37°C experimental plates showed CPE, while MDCKs infected with infected IPAM supernatants from 41°C-incubated plates showed no sign of infection. Only after 72 hours of infection was CPE visible on MDCKs infected with the supernatant from 41°C-infected IPAMs.

The last human virus examined, SouthCarolina/1918, displayed the most distinct temperature preferences from the WS/33 virus. Significantly lower titres at 33°C compared to 37°C were found at 24 and 48 hour time points ($p<0.05$), while no statistically significant differences were noted between 37°C and 41°C titres (Figure 16). Because the cells were infected at a specific MOI rather than receiving a set amount of plaque forming units, this resulted in higher overall titres at both experimental temperatures during some experiments while others had lower overall titres in both of the test conditions. In the end, this led to a lack of statistical significance, though evidence from individual experiments clearly demonstrated that the virus replicated equally well at 37°C and 41°C. Overall, this virus replicated to low titres similar to HongKong/68 virus, and likewise no hemagglutinating ability was found in any of the infected supernatants.



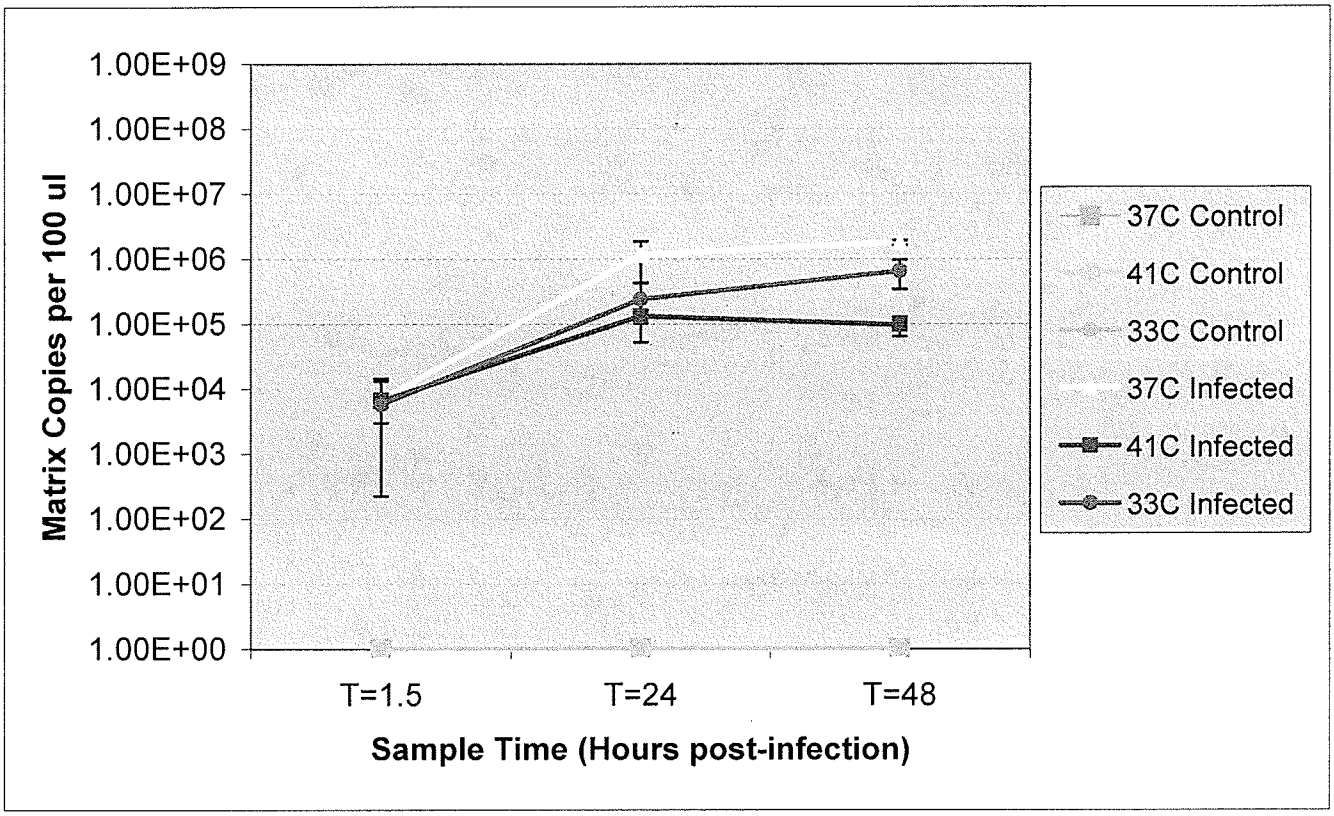
Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.233	0.996
41	37	0.0001	0.004
33	41	0.001	0.006

Figure 13. Growth properties of A/WS/33 (H1N1) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



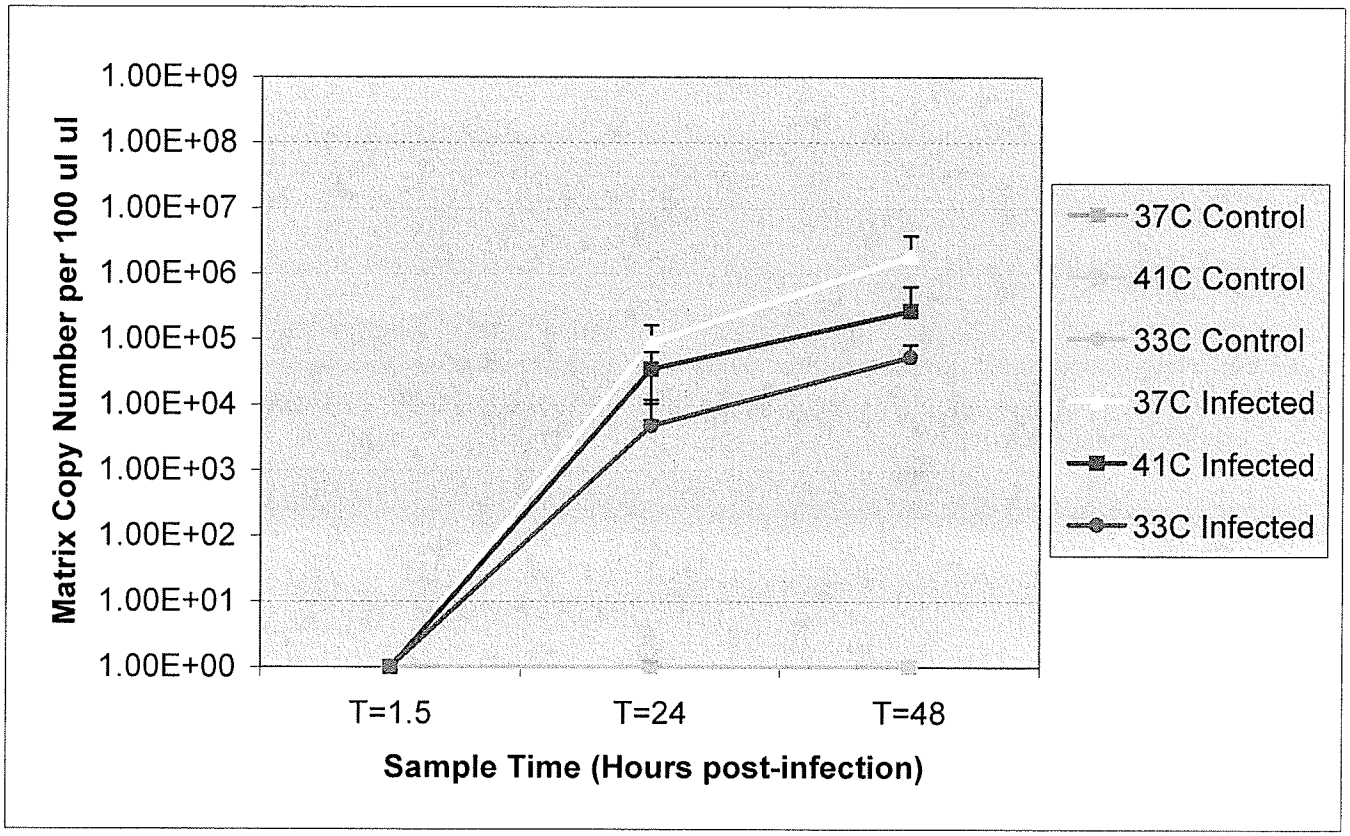
Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.018	0.198
41	37	0.011	0.021
33	41	0.915	0.151

Figure 14. Growth properties of A/PuertoRico/8/34 (H1N1) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.123	0.13
41	37	0.052	0.001
33	41	0.783	0.008

Figure 15. Growth properties of A/Hong Kong/8/68 (H3N2) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.



Incubation Temp		P-Value	
Temp 1	Temp 2	24hrs PI	48hrs PI
33	37	0.022	0.016
41	37	0.929	0.094
33	41	0.072	0.596

Figure 16. Growth properties of A/South Carolina/1918 (H1N1) in IPAMS over a 48-hour time-course. Cells were infected at an MOI of 0.07 and incubated at 33°C (blue line), 37°C (yellow line), or 41°C (red line). P-values of ≤ 0.05 indicate a significant difference when viral RNA levels were compared from cells incubated at two different temperatures at 24 or 48 hours post-infection (PI). Data are the mean (+/- standard deviation) of at least three independent experiments, and statistical analyses were conducted using a one-way ANOVA and Tukey's Honestly Significant Difference Test.

3. Influence of PB2 Sequences on Temperature Preference of Influenza Viruses in IPAMs

3.1. Residue 627

The amino acid at position 627 of PB2 did not influence influenza virus replication in the present system, and this was particularly evident in three cases. The Emu/Texas virus contained a lysine at residue 627, but showed typical avian-like temperature preferences, with RNA levels over 2 logs higher at 41°C compared to 33°C at 24 and 48 hours post-infection. Similarly, the SouthCarolina/1918 virus contained a lysine at this position but reached higher RNA levels at 41°C than 33°C. Conversely, the Swine/Texas virus contained a glutamic acid at this position, yet maintained a preference for 33 °C throughout the time course. Comparative replication rates of all experimental isolates at 33 °C and 41°C, highlighting their amino acid at residue 627, are found in Figure 17 (RNA levels at 24 hours post-infection) and Figure 18 (RNA levels at 48 hours post-infection).

3.2. Contribution of Eighteen Species-Specific Signature Amino Acids

To examine whether PB2 sequences of experimental isolates determined the behaviour of each virus at the different temperatures, eighteen amino acid residues were compared based on their suggested role in host adaptation and/or temperature sensitivity (Figure 19) (Miotto *et al.,* 2008; Steel *et al.,* 2009). In the case of the avian viruses, the possession of almost entirely avian-associated PB2 residues correlated with temperature preference. All replicated to high titres at 41°C and contained entirely avian-associated residues, with the exception Emu/Texas, which contained a single human-associated

residue. However, some viruses were better able to replicate at the lower temperatures than others, such as the Chicken/BC and the Duck/Albterta virus, which had generalized CPE and equal HA titres at all three temperatures by 48 hours post-infection. The temperature preferences of the human isolates also seemed to correlate with the number of human or avian-associated PB2 residues. The oldest human virus, SouthCarolina/1918, contained 13 avian-associated amino acids, and showed a phenotype more similar to avian viruses in terms of its temperature preference. The other two H1N1 human viruses each contained seven avian-associated amino acids and had a preference for 33°C and 37°C, but varied greatly in their ability to replicate at 41°C. The HongKong/68 virus contained mostly human-associated residues, with the exception of 701D, and also showed decreased replication at the higher temperature. The temperature preference of the swine viruses, however, did not correlate as well to their PB2 sequences. The Swine/1930 virus possessed the same number of avian and human-associated PB2 residues as the SouthCarolina/1918 virus, yet its replication rates were severely decreased at 33 °C and 41°C compared to 37°C. The Swine/Texas isolate had the poorest correlation between PB2 sequence and temperature preference, as it possessed an avian-like PB2 sequence with only a single human-associated amino acid, yet it replicated best at 33°C and worst at 41°C (Figure 18, Figure 19). PB2 sequences generated from in-house sequencing reactions are found in Figure 20, and sequences obtained from GenBank are found in Appendix IV.

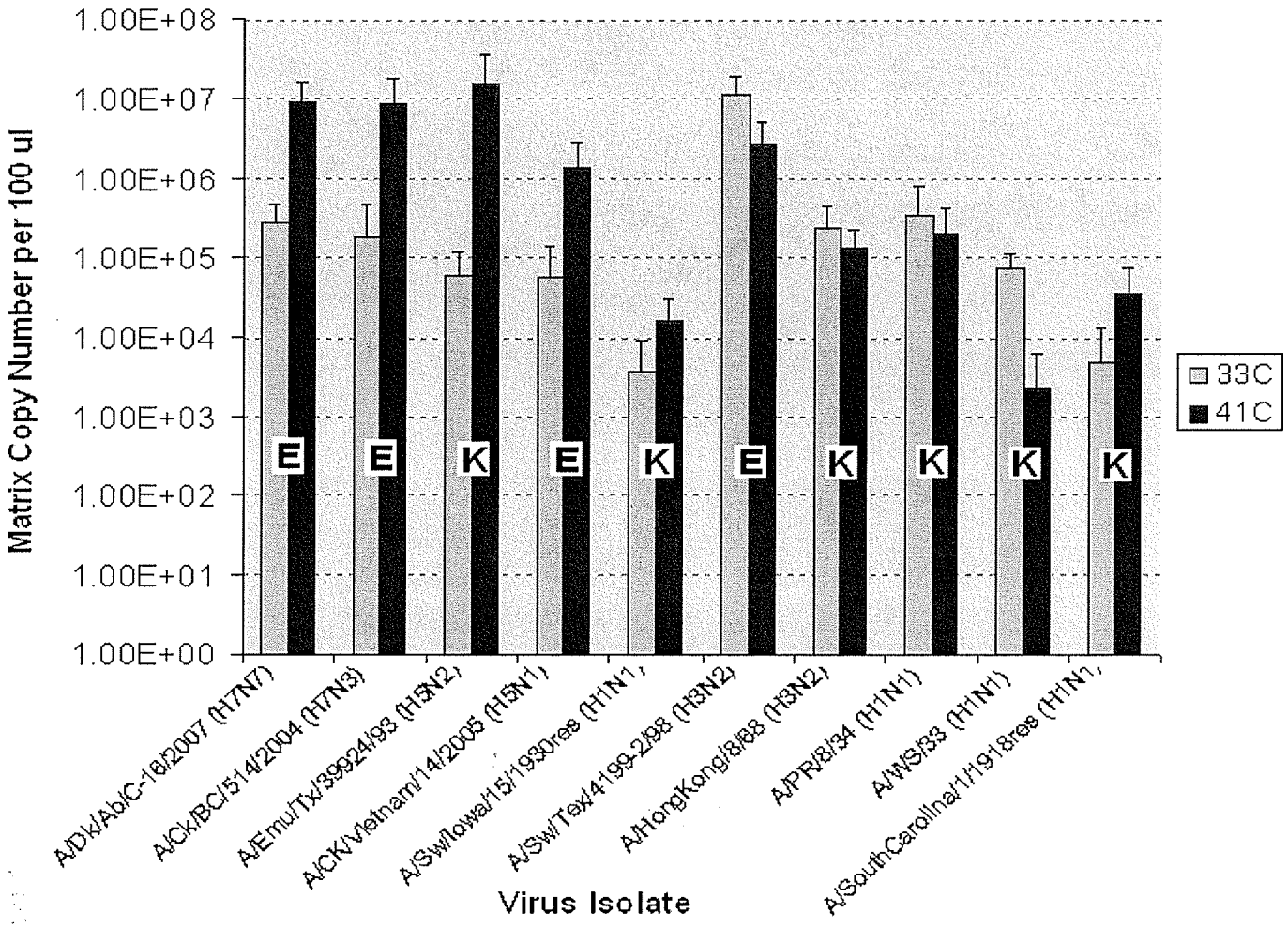


Figure 17. Growth of four avian, two swine, and four human influenza A viruses in IPAMs following 24 hours of infection at 33C (blue) and 41C (red) using a multiplicity of infection of 0.07. Values represent cumulative means (+ standard deviation) of at least three independent experiments at each temperature. PB2 residue 627 of each virus is indicated on the corresponding bars.

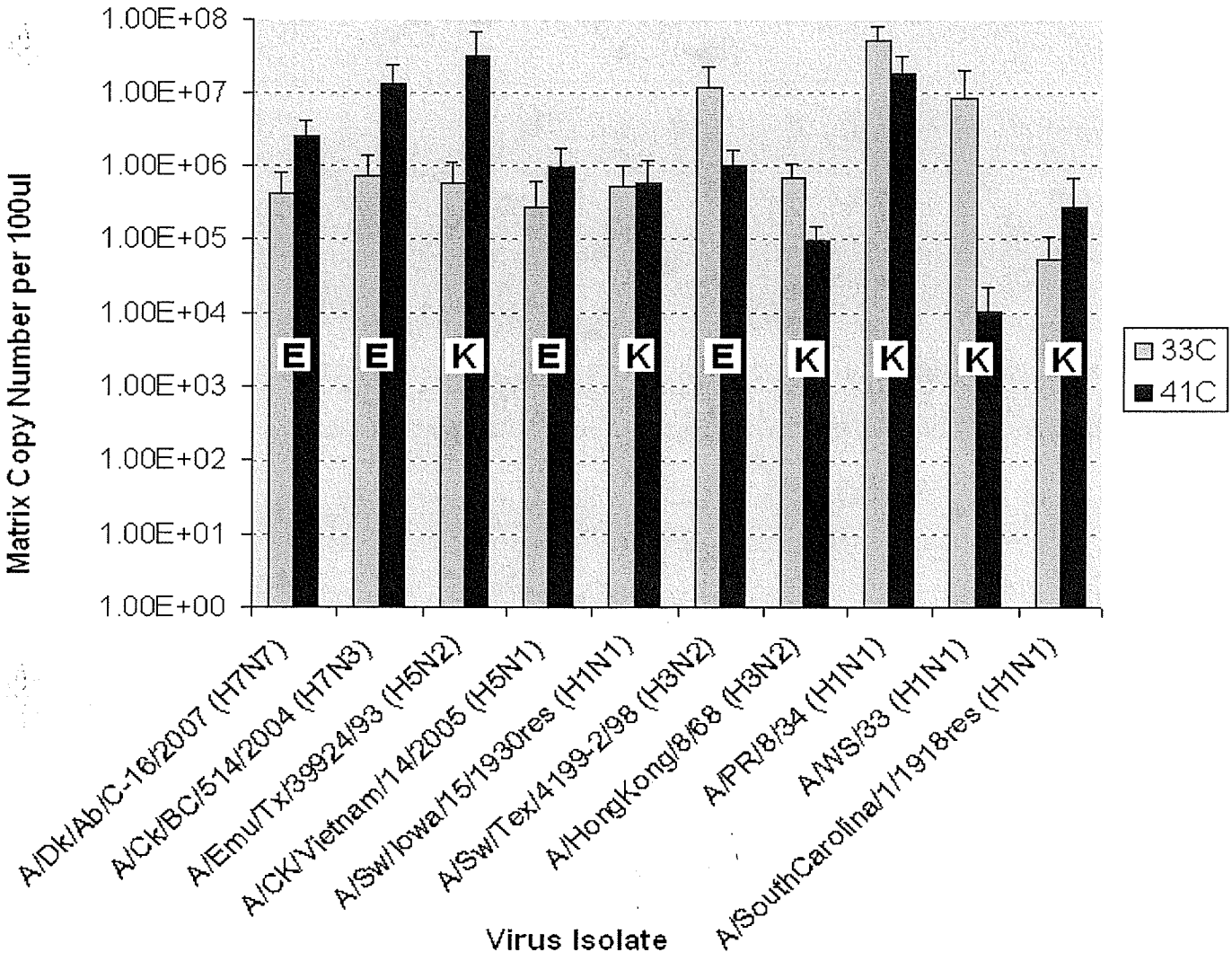


Figure 18. Growth of four avian, two swine, and four human influenza A viruses in IPAMs following 48 hours of infection at 33°C (blue) and 41°C (red) using a multiplicity of infection of 0.07. Values represent cumulative means (+ standard deviation) of at least three independent experiments at each temperature. PB2 residue 627 of each virus is indicated on the corresponding bars.

PB2 Residue

Virus Isolate	9	44	64	81	105	199	271	292	368	475	567	588	613	627	661	674	701	702
Duck/Alberta	D	A	M	T	T	A	T	I	R	L	D	A	V	E	A	A	D	K
Chicken/Vietnam	D	A	I	T	A	A	T	I	R	L	D	A	V	E	A	A	D	K
Emu/Texas	D	A	M	T	T	A	T	I	R	L	D	A	V	K	A	E	D	K
Chicken/BC	D	A	M	T	T	A	T	I	R	L	D	A	V	E	A	A	D	K
Swine/Iowa	D	A	I	T	T	S	T	I	R	M	D	A	V	K	T	A	D	R
Swine/Tex	D	A	M	T	T	A	A	I	R	L	D	T	V	E	A	A	D	K
SouthCarolina/1918	D	A	M	T	T	S	T	I	R	M	N	A	V	K	A	A	D	R
WS/33	N	A	T	M	V	S	T	I	R	M	N	V	A	K	T	T	D	R
PR8/34	N	A	T	M	I	S	A	I	R	M	N	I	A	K	A	T	D	K
HongKong/68	N	S	T	M	M	S	A	T	K	M	N	I	T	K	T	T	D	R

Avian-to-avian transmission associated

Human-to-human transmission associated

No species-specific association

Figure 19. PB2 sequence data of ten experimental isolates highlighting the amino acids at eighteen residues of suggested importance in sustained human-to-human transmission (green) or avian-to-avian transmission (purple) of influenza A viruses. Sequence data were obtained from in-house sequencing reactions or GenBank entries, and full-length PB2 sequences of each isolate are listed in Appendix XII.

Table 6. Cycle-Sequencing results for Emu/Texas, Swine/Iowa, SouthCarolina/1918, Chicken/BC, Chicken/Vietnam, and Duck/Alberta virus isolates from in-house sequencing reactions performed at either the National Centre for Foreign Animal Disease in Winnipeg or in the laboratories of collaborating investigators at the USDA in Ames, Iowa (Swine/Iowa) or the Mount Sinai School of Medicine in New York (SouthCarolina/1918). Residue numbers indicated on the left correspond to the first amino acid in each row of sequence data. Note that the Swine/Texas sequence begins at residue 40.

A/Chicken/BC/514/2004 (H7N3)

```
1   merikelrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  krimeiiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttstvh ypkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdtnpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckiap lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkr eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdmtps temslrgirv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkaarg qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappeqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dagaltedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Duck/Alberta/C-16/2007 (H7N7)

```
1   merikelrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  krimemiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttstvh ySkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitRekk eelqgckiap lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnVvrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkr eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdmtps temslrgirv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkaarg qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappeqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dagaltedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Chicken/Vietnam/14/2005 (H5N1)

```
1   merikelrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  kriiemiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpatsavh ypkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckiap lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkk eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd qqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdmtps temslrgvrv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
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541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkaarg qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappeqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dagaltedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Swine/Texas/4199-2/98 (H3N2)

```
1                                                  e knpalrmkwm mamkypitad
61  krimdmiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttstvh ypkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdtnpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckiap lmvaymlere lvrktrflpv aggtgsvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra avsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkk eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakalfqnwg iepidnvmgm igilpdmtps temslrgirv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkatrs rysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappeqsr mqfssltvnv rgsglrilvr gnspvfnynk
661 atkrltvlgk dagaltedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd s
```

4. Induction of Pro-inflammatory Cytokines in Infected IPAMs

After 24 hours of infection with all experimental isolates at 37°C, supernatants from infected IPAMs showed no production of TNF-α, IL-1β, or IFN-a, as determined by quantitative cytokine ELISAs. IPAM supernatants tested after 48 hours of incubation at 37°C had no detectable levels of IL-1β or IFN-α. A strong TNF-α response, resulting in ≥200 pg/ml, was induced by WS/33, HongKong/68, and Swine/Iowa. The Swine/Iowa isolate induced the strongest response by far, with an average of over 900pg/ml of TNF-α detected in infected samples. No significant levels of TNF were induced in response to the other two human viruses or the other swine virus, and none of the avian viruses induced any TNF-α in the infected IPAMs (Figure 20).

5. TNF-α Induction in IPAMS infected with 1930:1918HANA Recombinant Virus

To examine the influence of HA and NA on the induction of TNF-α, IPAMs were infected with a recombinant virus possessing the HA and NA of a non-TNF-α inducing virus, the SouthCarolina/1918 (H1N1) isolate, in the backbone of a strong TNF-α inducing virus, Swine/Iowa. Infection of IPAMs with the recombinant virus resulted in a significant decrease in TNF-α production compared to levels induced by the Swine/Iowa isolate, but not a complete loss. Infected cells produced a mean of 133.6pg/ml of TNF-α (+/- 40.85 standard deviation) compared to 0 pg/ml during a SouthCarolina/1918 infection or 904 pg/ml during a Swine/Iowa infection (Figure 20).

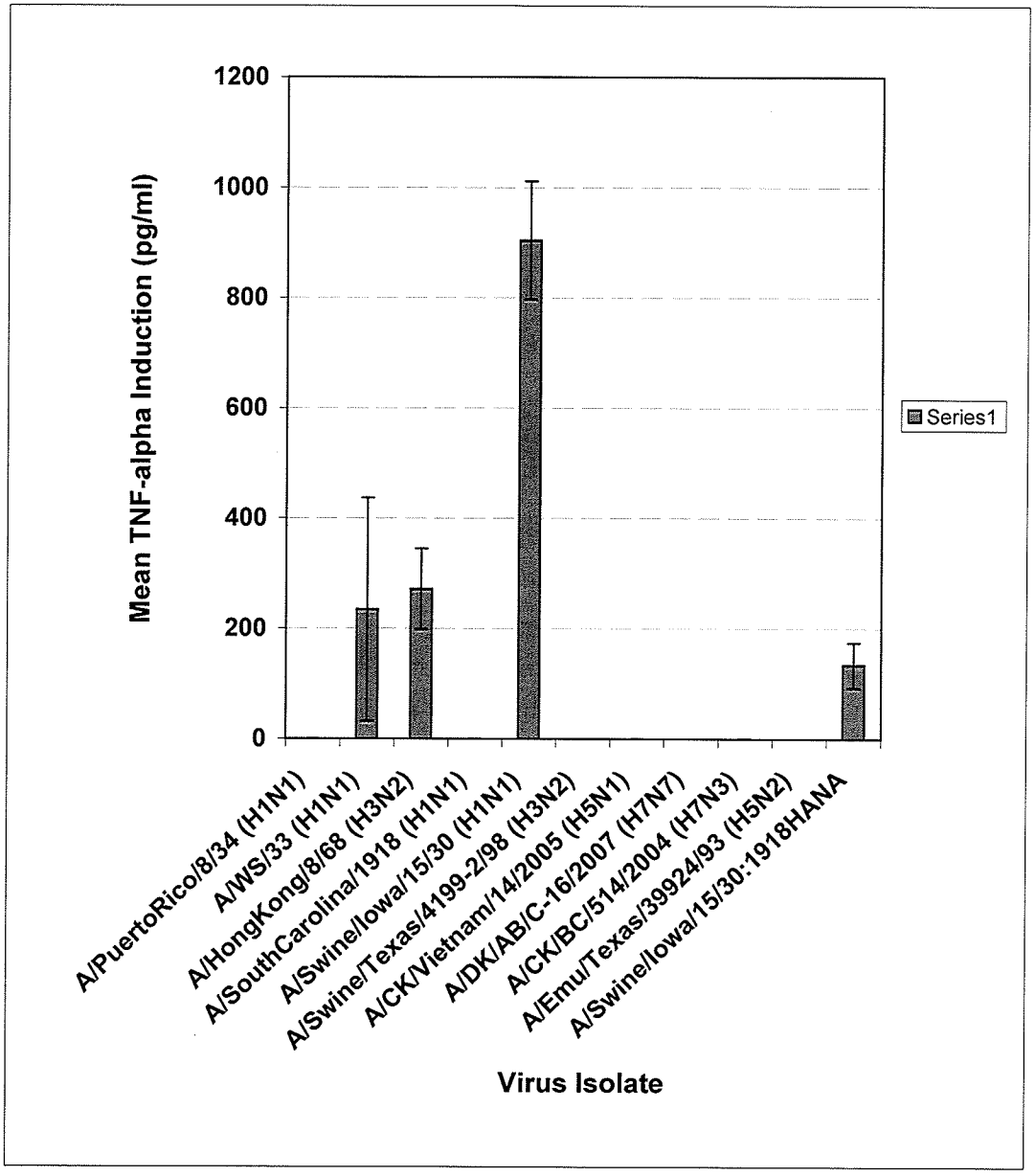

Figure 20. TNF-a induction in influenza A virus-infected IPAMs for 48-hours at 37C. Data were generated using a quantitative cytokine ELISA kit are the mean and standard deviation of quantitative cytokine ELISA results from two independent experiments, each with four replicates.

6. Virus Rescue

Attempts to rescue a virus with the HA and NA from the Swine/Iowa virus into the backbone of SouthCarolina/1918 were unsuccessful. All 12 plasmids were successfully amplified in the XL-10 gold cells; the presence of the desired insert was confirmed first by restriction enzyme analysis and finally by automated sequencing reactions (data not shown). Different lines of 293T cells and MDCK cells, along with two different transfection reagents, were used in an attempt to rescue the virus. Rescue plasmids for a control virus, A/PR/8/34, which was also provided in a 12-plasmid system, were included during each rescue trial. Rescues of the A/PR/8/34 virus were successful on every attempt regardless of the specific line of 293T cells or transfection reagent used. The sequence of the pPol I vector used for the PR/8 virus was approximately 1000 base pairs longer than the vector used for the SouthCarolina/1918 virus, which may have affected the efficiency of the rescues. Additionally, the PR/8 plasmids were provided in a ready-to-use condition, and it is possible that the DNA preparations for those plasmids were of higher purity that the plasmid preps done in-house for the recombinant virus rescue.

Discussion

The ability of a virus to replicate under physiological conditions atypical of its natural host has extremely important implications with regard to interspecies transmission. If certain groups of viruses are found to replicate more easily in a new host, and if there are specific molecular makers through which these viruses are identifiable, targeted surveillance programs can be set up to identify such agents. Pigs in particular are a concern for the generation of recombinant influenza viruses with the potential to infect new populations. For these reasons, a panel of influenza viruses was selected for testing in a swine *in vitro* model to characterize their ability to replicate under different conditions. Based on published findings by other groups, it was expected that viruses would replicate best under conditions most similar to their natural hosts, and that the amino acid at residue 627 of the PB2 gene would be a determining factor.

The results presented in this research describe, for the first time, the replication of influenza viruses at different incubation conditions in a swine *in vitro* system. The inclusion of ten viruses from avian, human, and swine host backgrounds provided an adequate sample size from which conclusions could be drawn about the abilities of different influenza virus lineages to successfully replicate under different physiological temperatures. Unlike observations from human or other mammalian systems, and in disagreement with the hypothesis, the swine *in vitro* system provided an environment where certain viruses could overcome temperature-mediated growth restrictions. Further, the nature of PB2 residue 627 did not determine temperature preference of a given virus in the present system. Looking at RNA replication alone, the PB2 amino acid signatures could predict the temperature preference of most viruses, with the exception of those

isolated from swine. However, when combined with CPE and HA results, neither species of isolation nor PB2 sequences could consistently predict the behavior of viruses at different temperatures.

The Chicken/BC virus was one such isolate that showed no major preference for the higher avian-like temperatures over the cooler human-like temperatures by 48 hours post-infection. From an epidemiological perspective, this virus was isolated from a highly pathogenic outbreak that lead to the rapid death of thousands of chickens and the culling of millions more. In addition to the poultry infections it caused, two human cases of conjunctivitis have occurred (Hirst *et al.,* 2004). The low temperature of the conjunctiva, due to constant contact with the air, is thought to act as a protective mechanism against infections (Al-Saimary, 2007). Therefore, the ability of the Chicken/BC virus to establish an infection at such an anatomical site coincides with the present results and provides insight on the natural ability of this virus to replicate at lower temperatures. However, the predilection of the Chicken/Vietnam virus for higher temperatures calls into question whether the ability of an avian virus to cause such human infections is a good indicator of its temperature preference. Viruses closely related to the Chicken/Vietnam isolate were responsible for a number of human infections in Asia in recent years; however this virus maintained a preference for high temperatures throughout the time-course experiment. Interestingly though, these infections were thought to occur deep in the lungs of infected patients rather than the upper respiratory tract, and were also associated with development of high fever (Tran *et al.,* 2004). The fact that two of the four avian viruses did not show a significantly different preference for any of the three experimental temperatures was quite surprising, as work by other

investigators found consistent differences in avian virus replication when grown at different temperatures in mammalian systems including canine and human cell lines as well as *in vivo* in mice (Hatta *et al.,* 2007; Labadie *et al.,* 2007; Massin *et al.,* 2001). It is possible that the swine cell factors, especially those that interact with influenza virus components during replication, are inherently more stable over a range of temperatures compared to other mammalian cells.

On initial phenotypic examination, the Emu/Texas virus appeared as a virus with temperature preferences similar to that of other avian viruses in *in vitro* systems. However, upon examination of its PB2 gene, this virus became much more interesting due to its preference for higher temperatures and possession of a lysine at residue 627. Lysine at this position was traditionally considered a human characteristic, rarely seen in wild bird isolates (Subbarao *et al.,* 1993; Massin *et al.,* 2001). However, recent data suggests that this is not necessarily the case. A 2009 publication on the molecular characterization of H5N1 viruses isolated from nine African countries showed that all isolates, from both wild and domestic birds, contained the E627K marker in their PB2 genes (Cattoli *et al.,* 2009). In fact, this trend has been noted in other reports on the descendents of the H5N1 Qinghai Lake virus outbreak (Hatta *et al.,* 2007). The possible advantage that this mutation may contribute to the viruses is unknown, however it is a clear demonstration that lysine at position 627 confers no disadvantage to virus replication and transmission in the avian host. This is also in agreement with work by Hatta and colleagues, who showed that replication of avian viruses in avian cells was equal at temperatures ranging from 33°C to 41°C regardless of whether they possessed a lysine or glutamic acid at PB2-627 (Hatta *et al.,* 2007). It is likely that given the role of

this residue in adaptation to mammalian species, it confers some competitive advantage to viruses even when maintained in avian populations.

The fact that the Emu/Texas virus was isolated from a ratite host (a group of large, flightless birds) also raises some interesting questions as to whether these types of birds induce a higher degree of mutations in the influenza viruses that infect them due to the fact that they are so different from typical wild aquatic bird hosts both phenotypically and physiologically (Capua 2009, verbal communication; Sales, 2005). Of the complete PB2 sequences in online databases (GenBank and GISAID) from rhea, emu, and ostrich isolates, half contain a lysine at residue 627 (See Appendix V for Accession numbers), and several published reports have indicated this mutation likely plays a role in adaptation to a new host (Subbarao *et al.,* 1993; Hatta *et al,.* 2007). Just as chickens provide an environment where highly pathogenic isolates emerge, it is possible that ratites present an environment that selects for mutations in PB2-627, though the reasons for this are unknown and further surveillance work is warranted in order to validate this assumption. Also of interest is that of the limited reported isolations of influenza viruses from ratites, the isolates that caused illness and death in these birds were determined to be of low pathogenicity for chickens (Alexander, 2000). This is another indication that there is something unique about these species that makes them even more sensitive to influenza virus infections than other types of domestic poultry.

One of the most interesting avian isolates was the Duck/Alberta virus due to its ability to reach high titres at 33°C. This virus was isolated from a group of ducks found dead near Edmonton, Alberta in February of 2007, though their deaths were ruled weather-related and not attributed to influenza after testing (Pasick *et al.,* unpublished

data). Assumedly, therefore, this virus had never circulated outside of its natural host species and should display characteristics of an avian virus in its evolutionarily static form. For such a virus to show replicative fitness at low and high experimental temperatures indicates that species of origin is not an indicator of how a virus will behave at different temperatures. Furthermore, given that it bares entirely avian-associated PB2 amino acid signatures; this indicates the importance of other viral proteins and host factors in determining temperature sensitivity in addition to PB2 determinants.

The growth properties of swine viruses at different incubation temperatures had not been previously reported in any *in vitro* systems. As the core temperature of pigs sits between 38.9-39.5°C (Refinetti, 2006), in between the human and avian core temperatures, it was hypothesized that these viruses should have a temperature preference similar to that of human viruses, as they too replicate over a range of temperatures in the upper and lower respiratory tracts. This was the case for the Swine/Iowa virus, which reached significantly higher titres at the intermediate temperature of 37°C compared to 41°C or 33°C. Sequence analysis of its PB2 gene also correlated with these results, and the fact that it contained a high number of avian-associated residues may explain why the virus did not grow as well at 33°C, though other contributing host and viral factors are likely involved.

Unlike the results for the Swine/Iowa virus, the growth properties of the Swine/Texas virus were not as expected, as it reached the highest titres at the coolest temperature rather than at 37°C or 41°C. These results were even more surprising after sequence analysis, as it contains an avian-acquired PB2 gene with exclusively avian-associated residues. However, the fact that this virus contains an NP segment from a

swine virus (Vincent et al., 2008) may contribute to its preference for lower temperatures, as the PB2-NP interaction was recently shown to be temperature-sensitive (Ramiex-Welti, 2009).

The SouthCarolina/1918 isolate was yet another example of a virus that did not conform to the assumptions on how a virus isolated from a specific host species should behave at different temperatures. Unlike the three other human viruses examined, this virus consistently displayed a preference for 41°C over 33°C. While these results were not found to be statistically significant, this was likely due to the fact that means are based on the results of six separate experiments, half comparing growth at 37°C and 33°C and the other half comparing growth at 37°C and 41°C. During all individual experiments, a significant difference was consistently observed between titres of 37°C and 33°C incubated cells, while experiments comparing 41°C and 37°C did not result in such differences (data not shown). From a genetic perspective, however, the PB2 sequence of this virus indicates that it is of avian origin, and the combination of avian-associated residues with the PB2-627K may explain its predilection for higher temperatures, much like the Emu/Texas virus.

The difference in final titres between the PR/8 and WS/33 viruses was quite interesting due to the fact that both are H1N1 viruses of human origin, isolated only one year apart and contain six PB2 residues associated with avian-to-avian transmission. Though the final PR/8 titres measured by RT-PCR indicated a significant difference between 37°C and 41°C, this difference was clearly not as biologically significant as for the WS/33 virus. Comparisons of the PB2 sequences from these viruses points to two avian-associated amino acids unique to the PR/8 virus, residues 661 and 702. The

recently resolved crystal structure of PB2 indicates that residue 661 is located on the same region as residue 627, known as the 627-domain. This region forms a highly ordered structure and contains a surface-exposed area, on which residues 627 and 661 are both located (Tarendeau *et al.,* 2008). While no influential role on temperature sensitivity has been ascribed to position 661, the fact that it is located in the same surface-exposed domain as residue 627 indicates that the nature of PB2-661 may influence the structure of the domain, possibly with a direct effect on residue 627.

In addition to the role of PB2-627 in determining transmissibility of influenza viruses in mammals, recent data has shed light on compensatory mutations in other PB2 residues that increase polymerase activity in mammalian cells. One particular amino acid that has received such attention is PB2 residue 701, which, when mutated from aspartic acid to asparagine, compensated for PB2-627E and allowed for efficient transmission of influenza virus between guinea pigs. Additionally, it played a role in decreasing temperature sensitivity and increased plaque size in MDCKs at 33°C. However, the degree to which this compensatory mutation affected plaque size was variable when isolates of different genetic backgrounds were compared, indicating other viral and cellular factors were at hand (Steel *et al.,* 2009). At the molecular level, the PB2 701N mutation was shown to strengthen the interaction between PB2 and importin alpha in mammalian cells, increasing nuclear accumulation of PB2 (Gabriel *et al.,* 2008). These data clearly demonstrate that, in agreement with the results from the present experiments, PB2 residue 627 is not the exclusive determining factor of host adaptation or temperature sensitivity, and that other amino acids contribute to these phenomena. Additionally, this also shows that the effects may vary between different isolates, as was found with the

present experimental isolates in the swine *in vitro* system. In the present study, PB2 residue 701 was unlikely to have played a role in determining temperature sensitivity of the experimental isolates as all viruses contained asparagine at this residue. However, it is reasonable to assume that other PB2 residues, yet to be described, also affect the ability of influenza A viruses to replicate at different physiological conditions.

New evidence suggests that the growth restriction of avian viruses in human cells is not a result of decreased association of PB2 with cellular RNA polymerase, but rather a decreased association of PB2 with NP. Recent studies have indicated the stability of molecular interactions between PB2 and NP are important in determining viral fitness in a new host, and that these interactions are influenced by incubation temperature (Labadie *et al.*, 2007; Rameix-Welti *et al.,* 2009). Therefore, while PB2 alone may influence this interaction in certain situations, the role of specific NP residues in stabilizing such interactions warrants further examination. Although differences in PB2 sequences of the experimental isolates from the present research do not point to specific residues involved in temperature sensitivity, NP sequences may provide clues into other determinants that have yet to be discovered. The possibility that this holds true for other mammalian systems exists, but caution must be exercised before automatically assuming the phenomenon is true for a swine system simply because it occurs in human cells. Data from this research and the work of others have highlighted a number of differences in the virus-host cell interaction when pig and human cells are compared, and it is reasonable to assume that these differences also exist in the factors affecting temperature sensitivity.

The fact that some isolates demonstrated decreased replication rates at certain experimental temperatures while other isolates were unaffected is a clear indicator that

temperatures sensitivity is a true phenomenon for influenza viruses in a swine system. Given the variation in temperature preference of viruses from the same host species, and limited correlation with PB2 sequences in certain situation, the determinants of temperature sensitivity of influenza virus are clearly complex and likely involve a number of viral and host factors.

Results from this work indicate the importance of using a large sample number to make accurate conclusions about the behavior of specific viruses under various conditions. Had only two avian and two human viruses been used in this experiment, the conclusions drawn could have been completely different. Additionally, real-time RT-PCR, hemagglutination assays and CPE observations proved to be a valuable and necessary combination of tools to accurately report on the temperature sensitive characteristics of the experimental isolates. Such was the case for the Emu/Texas virus, which statistically showed no difference in titres at 33°C and 41°C by 48 hours post-infection according to RT-PCR results, however HA results and CPE evidence showed that there was a significant biological difference between titres at the two temperatures.

These findings may have a number of important implications with regard to the role of pigs in interspecies transmission of influenza virus. In two of the four avian viruses examined, no significant preference for higher growth temperatures of 37°C and 41°C over 33°C was demonstrated. Similarly, select human viruses reached high titres at the avian-characteristic temperatures. This may indicate that human and avian viruses are not only able to easily infect pigs due to the availability of specific sialic acid receptors, but also because temperature does not affect their replication in these animals.

In addition to their usefulness for studies on temperature-dependent replication of viruses from various host backgrounds, the IPAMs were also extremely valuable for studies on the swine innate immune response to influenza virus infection. Alveolar macrophages are critical in the protection of pigs from influenza virus infection (Kim *et al.,* 2008), and results from the present research are an important contribution to the body of data on these cells and the factors that affect their response to infection.

The kinetics of virus infection in alveolar macrophages differs when cells of different host backgrounds are compared, as demonstrated by comparisons of mouse and human macrophage infections with the same influenza isolates (Perrone *et al.,* 2008). This is likely to be influenced by several factors including the availability of receptors as well as the strength and speed at which the cells mount an immune response against the virus. Conversely, the degree to which cells release immunological mediators in response to infection is likely influenced by the replication rate of the virus. Literature on infections of swine macrophages showed that titres peak at 48 hours following infection with influenza virus (Seo *et al.,* 2004), and the same results were found in the present study. Preliminary time-course experiments were conducted over a longer period of time, with sampling over a 72-hour period, but since titres peaked by 48-hours post-infection, all future experiments were stopped at the 48-hour mark. Previous analyses of sialic acid receptors from freshly harvested swine alveolar macrophages revealed the presence of both α-2,6 and α-2,3-linked sialic acid species on the surface of these cells, with α-2,6 linkages present at higher concentrations (Seo *et al.,* 2004). Immunofluorescent detection of these molecules on the surface of IPAMs agreed with the published results, though the relative amounts of each receptor species were not quantified. However, even if IPAMs

contained a higher proportion of α-2,6 linkages, growth curves showed that avian and mammalian viruses were equally able to reach high titres in these cells, indicating receptor abundance was not a limiting factor in either case.

Numerous studies have shown that different influenza viruses induce different levels of cytokines following infection of monocytes or macrophages (Cheung *et al.*, 2002; Seo *et al.*, 2004). A previous publication indicated that the production of cytokines *in* vitro by swine alveolar macrophages was influenced by the percentage of infected cells and the surface glycoproteins of the infecting viruses; with high ratios of infected to uninfected cells leading to the production of several cytokines, and the production of TNF-α being up-regulated upon infection with H3N2 compared to H1N1 human viruses (Seo *et al.*, 2004). Results from the present experiment were not in complete agreement with these findings, as infection with the HongKong/68 (H3N2) virus induced only slightly more TNF-α than the WS/33 (H1N1) virus. Additionally, that the Swine/Texas virus did not induce any TNF-α production was quite surprising considering it also harbours H3N2 surface glycoproteins derived from human lineage viruses (Webby *et al.*, 2004). Results from the recombinant virus work also were not in complete agreement with published findings on the contribution of individual genes to TNF-α induction. Work by Seo and colleagues using single and double-gene reassortants showed that TNF-α induction was dependent on the HA and NA of influenza virus (Seo *et al.*, 2004). In the present study, replacement of the HA and NA of a strong TNF-α inducer (Swine/Iowa) with those from a non-inducing virus (SouthCarolina/1918) resulted in a significant decrease but not a complete loss of TNF-α production following infection.

This indicates that although the HA and NA had a major impact on TNF-α production, other viral gene products played an inducing role in the IPAMs following infection.

The fact that no IL-1β or IFN-α were detected in the infected supernatants came as a surprise since these cytokines were known to be important mediators in the swine response against influenza (Charley *et al.,* 2006; Van Reeth, 2000; Seo *et al.,* 2004). However, very few studies have examined the levels of these cytokines following *in vitro* infection of swine alveolar macrophages directly, and the one study that conducted such an investigation detected IL-1β at only very low levels by RT-PCR at an early time post-infection and not by ELISA (Seo *et al.,* 2004). The lack of IFN-α in infected supernatants may be the result of the NS1 protein, which is a known inhibitor of the type 1 interferon response (Wright *et al.,* 2006). While IFN-α production has been seen following *in vitro* infection with influenza virus in certain cell types, it is possible that IPAMs are particularly sensitive to the effects of NS1 and subsequently are unable to produce interferons during influenza infection, though work by other lab members has proven these cells are capable of producing IFN-α during infection with other viruses such as Nipah virus (H. Weingartl, unpublished data).

Another unexpected result was that none of the avian influenza viruses induced any cytokine production in the IPAMs. This could not be explained by final virus titres being too high or too low, as avian viruses reached final titres similar to those reached by the TNF-inducing human and swine isolates. Previous *in vivo* studies correlated the degree of TNF-α induction in the lungs of experimentally infected pigs with virus pathogenesis (Van Reeth *et al.,* 2002), but the same relationship was not observed here. The degree of CPE observed by 48 hours post-infection was not correlated with TNF-α

induction, as the Swine/1930 virus caused massive CPE in infected cells whereas the HongKong/68 virus caused limited CPE, and both of these viruses were TNF-α inducers. While the present data does not agree with published *in vivo* results, it is important to note that studies using tissue culture do not accurately reflect the *in vivo* situation, which is a likely explanation for the differences observed. It would seem that some other inherent property of these avian viruses allows them to escape a TNF-mediated response, but results from this work do not suggest the mechanisms responsible for this phenomenon.

Future Research and Conclusions

A number of future studies could be carried out to strengthen the results from this research and to provide insight into unanswered questions. To further investigate the theory that swine cells in general provide an environment where temperature sensitivity is decreased, a number of studies should be considered. Viruses should be tested for temperature sensitivity in other swine cell types from natural sites of infection, such as respiratory epithelial cells, to see whether the present results hold true in all influenza virus-susceptible swine cell types or whether these results are specific for alveolar monocytes/macrophages only. It would also be very interesting to see whether avian and human viruses with demonstrated temperature sensitivity in human cells could be successfully cultured in IPAMs or other swine cells under the same conditions. Detailed analyses of NP gene sequences from experimental isolates should also be undertaken in order to possibly distinguish molecular makers in this gene that might play a synergistic role with PB2 in determining temperature sensitivity. With regard to the induction of

TNF-α in IPAMs, the inclusion of a large panel of avian viruses for future studies is warranted to determine whether the lack of TNF-α induction is a true phenomenon for all avian viruses or merely a result of a small sample size.

Results from this work show that temperature preference of influenza A viruses cannot be assumed based on the host species from which the virus was isolated, and in particular that residue 627 of PB2 does not dictate an isolates ability to replicate at specific temperatures in a swine system. The factors that influence temperature sensitivity in the present system appear to be polygenic, with both viral and host genes likely involved.

The ability of both human and avian viruses to reach high titres in key cells of the swine immune system is a major cause for concern in terms of the role of pigs as mixing vessels for the generation of viruses with pandemic potential. This is further exacerbated by the fact that incubation temperature was not a limiting factor in the rate of replication for several viruses, indicating that isolates from varied host backgrounds may easily infect a swine system regardless of the core temperature of their natural host. IPAMs proved to be a valuable model of the swine innate response to influenza viruses as they secreted TNF-alpha in response to infection, and further investigations are warranted to determine why avian viruses did not elicit a response.

Taken together, these results contribute novel findings on the replication of influenza viruses and their ability to induce certain cytokines in a swine *in vitro* model, and emphasize the importance of using a holistic approach to address questions regarding virus-host interactions.

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Appendix I – Preparation of Buffers for RNA Extraction

RLT Buffer (Qiagen) – To 100 ml of kit-supplied RLT buffer, add 1.0 ml of betamercaptoethanol (β-ME) and mix by pipetting up and down until evenly dispersed. Add 100 ml of 70% EtOH (prepared with nuclease-free water) to the RLT-β-ME solution and mix well. The resultant solution was used for RNA extractions and was kept at room temperature for up to 30 days; at which time a new batch was made.

RPE Buffer (Qiagen) – 220 ml of 70% EtOH (prepared with nuclease-free water) was added to the kit supplied bottle of RPE concentrate (55 ml), resulting in 275 ml of RPE buffer used for RNA extractions.

Appendix II – Determination of gene copy numbers in plasmid DNA preparations for Real-time RT-PCR standard curve.

The standard curve for each real-time RT-PCR run was constructed using 10-fold dilutions of pPolI 1918 Matrix DNA. This DNA preparation was measured using a nanodrop spectrophotometer, and the resulting concentration was used to calculate the number of matrix gene copies in the preparation. The length of the plasmid and the length of the insert, in base pairs, was also required information for the calculation. The step-by-step process using a plasmid DNA preparation with a concentration of 546ng/µl was as follows:

$$\text{Copy number per } \mu l = \frac{\text{Avogadro's number} \times \text{plasmid DNA concentration [grams/µl]}}{\text{Molecular Weight [grams/mol]}}$$

1918 pPolI-Matrix plasmid – Concentration = 546 ng/µl = 5.46×10^{-7} grams/µl

pPolI vector length = 2911 bp

1918 Matrix gene length = 982bp

Total plasmid size (vector plus insert) = 3893bp

Weight in Daltons (g/mol) = (bp size of vector + insert) X (330 Da X 2 nucleotide/bp)

= 3893 X 660

= 2,569,380 (grams per mol)

$$\text{Copy number per } \mu l = \frac{6.02214199 \times 10^{23} \times 5.46 \times 10^{-7}}{2{,}569{,}380}$$

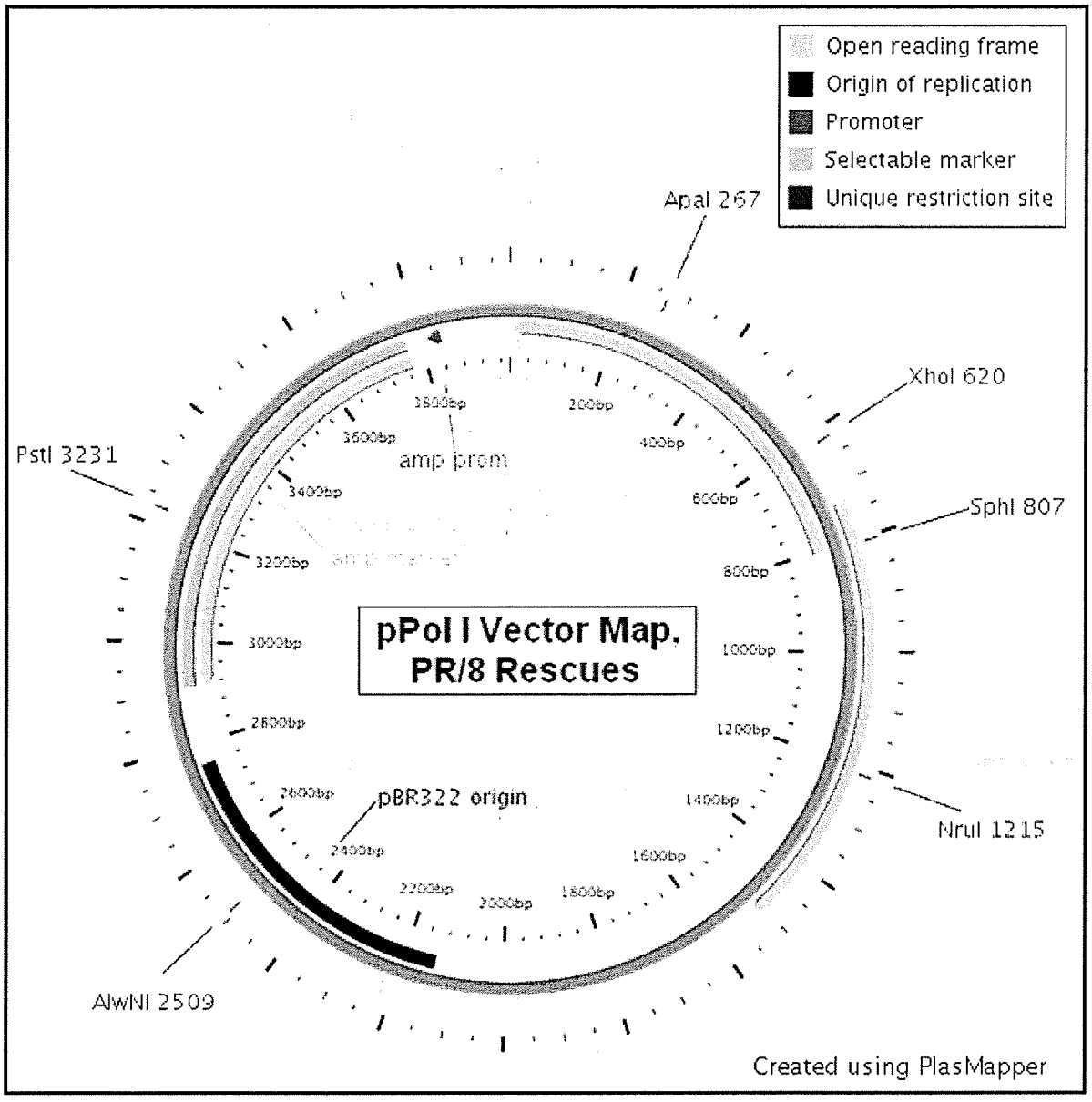
$$= \frac{3.28808 \times 10^{17}}{2{,}569{,}380}$$

$= 1.279 \times 10^{11}$ copies/µl in stock 1918 pPol I Matrix plasmid DNA preparation.

From this point, the sample was diluted with RNASecure to reach a concentration of 1×10^{10} copies/µl, and serial 10-fold dilutions were made from this 1×10^{10} solution and included in the real-time RT-PCR runs.

Appendix III –Map of PolI vRNA Expression Plasmid for PR/8 Virus

Data for the PR/8 virus pPol I vector was provided by Dr. Darwyn Kobasa at the National Microbiology Laboratory in Winnipeg. The map was generated by entering the 3980bp sequence onto the PlasMapper (Version 2.0) server accessed online through http://wishart.biology.ualberta.ca/PlasMapper/ (Dong *et al.*, 2004). Note that only unique restriction sites are indicated.



Appendix IV – PB2 Protein Sequences of Experimental Isolates Obtained From GenBank Entries.

A/Emu/Texas/39924/93 (H5N2) GenBank accession # ACF25081

```
1   merikelrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  kkimemiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttstih yprvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckiap lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkk eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm vkavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg ietidnvmgm vgilpdmtps temslrgirv
481 skmgvdeyss tervvvnidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkaarn qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappkqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dageliedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Swine/Iowa/15/1930 (H1N1) GenBank accession # AAA43126

```
1   merikelrdl msqsrtreil trttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  kriietiper neqgqtlwsk tsdagsdrvm vsplavtwwn rngpttstvh ypkiyktyfe
121 kaerlkhgtf gpvhfgnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqlmitkekk eelqdckisp lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqlytpggev rnddvdqsli iaarsivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkk eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr lvqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 inranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdltps temsmrgvri
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwevn
541 gpesvlvnty qwiirnwetv kiqwsqdptm lynkmefepf qslvpkaarg qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappkqsr mqfsslavnv rgsgmrilvr gnspvfnynr
661 ttkrltvlgk dagaltedpd egttgvesav lrgflilgke drrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Swine/Texas/4199-2/98(H3N2) GenBank accession # AAD51248

```
1         lrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  krimdmiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttstvh ypkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdtnpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckiap lmvaymlere lvrktrflpv aggtgsvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra avsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkk eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdmtps temslrgirv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgt
```

A/South Carolina/1918 (H1N1) GenBank accession # ABA55038

```
1   merikelrdl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  krimemiper neqgqtlwsk tndagsdrvm vsplavtwwn rngpttsavh ypkiyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckisp lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkr eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iepidnvmgm igilpdmtps temsmrgvrv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwevn
541 gpesvlvnty qwiirnwetv kiqwsqnptm lynkmefepf qslvpkaarg qysgfvrtlf
601 qqmrdvlgtf dtvqiikllp faaappkqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dagaltedpd egtagvesav lrgflilgke drrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Wilson-Smith/33 (H1N1) GenBank accession # ABD77806

```
1   merikelrnl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  kritemiper neqgqtlwsk mndagsdrvm vsplavtwwn rngpvtstvh ypkiyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqgckisp lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra tvsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkr eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedci ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iesidnvmgm igilpdmtps temsmrgvrv
481 skmgvdeyss aekvvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqnpam lynkmefepf qslvpkavrg qysgfvrtlf
601 qqmrdvlgtf dtaqiikllp faaappkqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 ttkrltvlgk dagtltedpd egtagvesav lrgflilgke drrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrn ssiltdsqta tkrirmain
```

A/Puerto Rico/8/34 GenBank accession # ABD77685

```
1   merikelrnl msqsrtreil tkttvdhmai ikkytsgrqe knpalrmkwm mamkypitad
61  kritemiper neqgqtlwsk mndagsdrvm vsplavtwwn rngpitntvh ypkiyktyfe
121 rverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckisp lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra avsadplasl lemchstqig girmvdilrq
301 npteeqavdi ckaamglris ssfsfggftf krtsgssvkr eeevltgnlq tlkirvhegy
361 eeftmvgrra tailrkatrr liqlivsgrd eqsiaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg vepidnvmgm igilpdmtps iemsmrgvri
481 skmgvdeyss tervvvsidr flrirdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqnptm lynkmefepf qslvpkairg qysgfvrtlf
601 qqmrdvlgtf dtaqiikllp faaappkqsr mqfssftvnv rgsgmrilvr gnspvfnynk
661 atkrltvlgk dagtltedpd egtagvesav lrgflilgke dkrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

A/Hong Kong/8/68 GenBank accession # ACF22286

```
1   merikelrnl msqsrtreil tkttvdhmai ikkytsgrqe knpslrmkwm mamkypitad
61  kritemvper neqgqtlwsk msdagsdrvm vsplavtwwn rngpmtstvh ypkvyktyfe
121 kverlkhgtf gpvhfrnqvk irrrvdinpg hadlsakeaq dvimevvfpn evgariltse
181 sqltitkekk eelqdckisp lmvaymlere lvrktrflpv aggtssvyie vlhltqgtcw
241 eqmytpggev rnddvdqsli iaarnivrra avsadplasl lemchstqig gtrmvdilrq
```

```
301 npteeqavdi ckaamglris ssfsfggftf krtsgssikr eeelltgnlq tlkirvhegy
361 eeftmvgkra tailrkatrr lvqlivsgrd eqsvaeaiiv amvfsqedcm ikavrgdlnf
421 vnranqrlnp mhqllrhfqk dakvlfqnwg iehidnvmgm igvlpdmtps temsmrgirv
481 skmgvdeyss tervvvsidr flrvrdqrgn vllspeevse tqgtekltit ysssmmwein
541 gpesvlvnty qwiirnwetv kiqwsqnptm lynkmefepf qslvpkairg qysgfvrtlf
601 qqmrdvlgtf dttqiikllp faaappkqsr mqfssltvnv rgsgmrilvr gnspvfnynk
661 ttkrltilgk dagtliedpd egtsgvesav lrgflilgke drrygpalsi nelsnlakge
721 kanvligqgd vvlvmkrkrd ssiltdsqta tkrirmain
```

Appendix V – List of published ratite isolates, amino acid at PB2 residue 627, and accompanying accession numbers for full PB2 sequences in GenBank.

Influenza A isolates from ostrich, emu, and rhea hosts. Where available, NCBI accession numbers for PB2 protein sequences are provided. Sequences of the two isolates without accession numbers were obtained from the GISAID (Global Initiative on Sharing Avian Influenza Data) online platform.

NCBI Accession Code	Isolate Name	PB2-627
AAQ04929	A/ostrich/South Africa/9508103/95(H9N2)	K
N/A	A/ostrich/Saudi Arabia/6732-3/2007 (H5N1)	K
N/A	A/ostrich/Denmark/96-72420/1996 (H5N2)	V
ABS50695	A/ostrich/Israel/1436/2003(H9N2)	E
ABJ16872	A/ostrich/Nigeria/1047-25/2006(H5N1)	K
ABO45258	A/ostrich/Italy/1038/2000(H7N1)	E
ABJ90222	A/ostrich/Italy/2332/00(H7N1)	K
ABJ90266	A/ostrich/Italy/984/00(H7N1)	E
ACF25628	A/emu/NY/12716/1994(H5N9)	E
ACG59937	A/emu/TX/25414/1995(H7N2)	K
ACF25081	A/emu/TX/39924/93 (H5N2)	K
ABR37450	A/emu/New South Wales/775/1997(H7N4)	E
ACF24954	A/rhea/North Carolina/39482/1993(H7N1)	E
ACF25657	A/rhea/TX/39923/1993(H5N2)	K