EVALUATION OF A NOVEL ANTIVIRAL FOR INFLUENZA INFECTION IN THE FERRET MODEL

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Allison Leigh Brichacek

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This thesis was presented

by

Allison Leigh Brichacek

It was defended on

April 7, 2016

and approved by

Amy L. Hartman, Ph.D.
Assistant Research Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Simon Barratt-Boyes, BVSc, Ph.D.
Professor
Department of Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

John F. Alcorn, Ph.D.
Associate Professor
Department of Pediatrics and Immunology
School of Medicine
University of Pittsburgh

Thesis Director: Kelly Stefano Cole, Ph.D.
Associate Professor
Department of Immunology
School of Medicine
University of Pittsburgh

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Kelly Stefano Cole, Ph.D.

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Allison Leigh Brichacek, M.S.

University of Pittsburgh, 2016

ABSTRACT

Influenza viruses, although common, cause thousands of deaths worldwide and remain a prominent public health issue, especially with the growing population of elderly and immune-compromised individuals. Currently, the influenza vaccine is the best tool available at protecting against infection, but the correct strains are hard to predict and the vaccines do not always work. Therefore, it is necessary to explore more effective antiviral drugs. New antivirals like these are of public health importance because they can help reduce morbidity and mortality related to influenza infection. Our collaborators from the University of Washington have computationally designed a small protein, HB36.6, which interferes with influenza infection by binding the virus' hemagglutinin surface protein. This thesis has tested the novel antiviral using the influenza A/California/07/09 (H1N1) strain in a ferret model by looking at the effects of low and high doses of HB36.6, and comparing it to untreated controls or against the current standard antiviral, Tamiflu. Antiviral effectiveness was evaluated using a combination of clinical and viral parameters. First, the animals were monitored and scored using a detailed clinical scoring system for onset of clinical signs of disease, as well as tracked through daily measurements of weights and temperatures. Second, viral titers were quantitated using RT-PCR in tissue samples obtained at necropsy and the untreated controls were compared to HB36.6-treated animals or those treated with Tamiflu. The overall hypothesis for this study was that HB36.6 will be

effective in reducing viral loads and limiting disease following aerosolized influenza infection in the ferret model, and this reduction in viral loads/disease would be more effective than the currently used antiviral, Tamiflu. The results of these studies show that HB36.6-treated animals display fewer clinical symptoms when a low dose of the treatment is used, but there appears to be no reduction in viral loads. Higher doses appear to be toxic to the animals, especially when taken over multiple days throughout the course of infection. These studies have yielded important information on a new class of influenza therapeutics that should be further evaluated using larger sample sizes.

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1.0   INTRODUCTION

Influenza is a negative-sense, single-stranded RNA virus consisting of three subtypes, A, B, and C. These viruses contain 8 segments that code for 11 or 12 proteins. Influenza virus is the only virus within the family *Orthomyxoviridae* that can cause infection in humans. Influenza A virus is the most virulent and can cause serious disease in humans, as well as birds and other mammals, while humans remain the only natural reservoir for Influenza B [1, 2]. Influenza viruses are classified according to their surface proteins, hemagglutinin (HA) and neuraminidase (NA). NA, a glycoprotein that cleaves sialic acid from host cell membranes to increase the likelihood of viral release, is highly conserved among influenza strains [3]. HA mediates viral binding to their target cells through recognition of the terminal sialic acid residues [4]. Influenza viruses are categorized into two phylogenic groups, which consist of 18 HA and 11 NA identified subtypes [5, 6]. Avian and swine Influenza A viruses are of great interest to researchers because of these animals proximity to humans (livestock and household pets), and therefore, opportunities to allow the disease to mutate and spread. Together, these viruses cause an average of 250,000-500,000 deaths annually around the world and infect 5-20% of the US population [1, 2, 5]. HA and NA periodically undergo antigenic drift (minor changes such as point mutations in the RNA gene segments), resulting in redirection of the immune response. Antigenic shift takes place when a host is infected with two different viruses and there is

reassortment of gene segments during viral replication, creating a new virus strain, which can cause pandemics [1, 2].

Influenza A viruses primarily target epithelial cells in the upper and lower respiratory tracts during infection, as the HA surface proteins bind to exposed sialic acid residue glycoproteins on the surface of respiratory epithelial cells. This protease-mediated cleavage promotes viral entry by activating endocytosis of the virus. A low pH in the endosome allows for uncoating of the virion so that it may undergo viral replication. Following viral replication, progeny are transported to the cell membrane to once again bind the sialic acid residues via NA. This time, the linking promotes viral release, so that the virus may move on to infect more cells [2]. Influenza infection, like many other diseases, causes an inflammatory response in the body, which activates both innate and adaptive immune responses. Normal response stages to inflammation include, "(i) recognition of the problem, (ii) recruitment of leukocytes and other immune system components, (iii) elimination of the threat, and (iv) resolution of the inflammatory state (i.e., a return to homeostasis)" [7].

In 2009, a novel influenza A H1N1 caused the first pandemic of this century as a strain with mixed avian, swine and human IV genes from North American and Eurasian swine influenza virus lineages. Compared to seasonal influenza, this pandemic strain is highly pathogenic with considerably more viral replication in the upper respiratory tract [8]. Today, the A(H1N1)pdm09 strain can still cause mild to severe disease resulting in hospitalization and mortality, and therefore remains a prominent public health issue [1].

1.1   BURDEN AND EPIDEMIOLOGY

To predict the next seasonal influenza strains, scientists watch the Southern Hemisphere countries, like New Zealand, where influenza season occurs between June and September. After the 2009 influenza pandemic, the US Department of Health and Human Services, Centers for Disease Control and Prevention (CDC) funded the Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) project to take place over five years, from 2012-2016, in New Zealand. The goal of this project is to measure influenza burden, epidemiology, etiology, risk factors, immunology, effectiveness of vaccination, and other prevention strategies for influenza. In the first year of the study, the overall adjusted annual incidence rate of hospitalization due to influenza was 54/100,000 persons, which is similar to US data from 1979-2001 showing an average annual incidence of 36.8/100,000 persons. It also confirmed that the very young ($\leq$4 years), elderly ($\geq$65), and the most deprived socio-economic status (SES) groups have the highest rates of influenza-associated hospitalizations [9].

Severe acute respiratory illness (SARI) surveillance often underestimates the true burden of influenza-associated hospitalizations and mortalities due to patients not meeting the case definition, or being incorrectly diagnosed, tested, and recorded as having influenza. When a patient dies of a secondary bacterial infection or exacerbation of a pre-existing condition, this is what is recorded on the death certificate. SHIVERS data on influenza-like illness (ILI) is used to measure disease burden of moderate influenza disease. These data show that preschool children (1-4 years), school-age children, adults (<65 years), people of Asian ethnicity, and those of lower SES are at greatest risk of developing ILI-associated influenza [9]. In the US, for the 2013-2014 influenza season there were more than 105 influenza-related child deaths reported to the CDC

and the vaccine was determined to only be 62% effective against the most prominent virus strain, 2009 H1N1 [10].

1.2   THE FERRET MODEL

Because of the impact of influenza viruses in the community, many researchers study the disease using animal models. In order to better understand influenza A infection in humans, researchers have used various animal models, including mice, ferrets, cats, dogs, guinea pigs, and nonhuman primates. Mice are one of the most commonly used models for influenza research because they are inexpensive, easy to house, easily accessible, and murine-specific immunologic reagents are widely available. However, mice are not a natural host for influenza viruses, so the strains must be adapted for mouse studies. Also, mice do not display clinical signs of disease in the same way as humans, and these signs can differ depending on the dose of virus administered [2].

Ferrets, *Mustela putorius furo*, have been used in influenza research since the 1930s [2, 5]. Their main advantages include a similar presentation of disease to human through observed clinical symptoms, they are naturally susceptible to human influenza viruses, and they can be used to study the different modes of influenza transmission [2]. Ferrets present similar clinical signs of disease as humans, including fever, nasal discharge, sneezing, and lethargy [2, 11]. They are also small to intermediate in size, making them convenient to house and transport, and they prefer to be caged in same-sex groups. In relation to smaller rodent models, ferrets can provide more sizable blood and tissue samples [5, 11]. The complete ferret genome and transcriptome can be found online to offer ways of comparing species-specific genes and

developing undiscovered signaling pathways. Ferrets can even be used to demonstrate and predict the emergence of drug-resistant strains of influenza [5].

Ferrets also share similarities to humans in the respiratory tract, lung physiology, airway morphology, and metabolism [1, 5]. Influenza A virus (IAV) presents similarly in the ferret and human respiratory tract because they both have a similar distribution of sialic acid receptors in the trachea and lower respiratory tract [2, 8]. One main difference is that influenza infection in ferrets rarely progresses to pneumonia because the viruses manifest in the upper respiratory tract rather than the lower respiratory tract. Ferrets are useful in vaccine research because they develop active immunity with neutralizing antibodies after being infected with human IAV strains [2]. Severity or fatality of disease can depend on the age of the host and the strain of virus [1].

Some limitations of the ferret model include their increased expense over mice, their limited commercial availability, the requirement for more complex housing arrangements, and the limited availability of reagents [2]. Ferrets are now being offered as specific pathogen free (SPF) by some commercial breeders, where they pre-test animals for infectious disease before purchase. However, due to the natural susceptibility of ferrets for human respiratory virus, it is highly recommended that individual laboratories confirm the serological status for disease of the ferrets prior to infection. Currently, there is limited availability of ferret-specific immunological reagents. Researchers are left to test and identify reagents from other species that may be cross-reactive for the ferret. Fortunately, using mRNA expression levels though quantitative real-time RT-PCR researchers are able to study innate immune responses in ferrets similarly to those seen in human patients [5]. Therefore, despite these limitations, ferrets remain an important model

for influenza research as they can provide a form of active surveillance on the pathogenicity and virulence of new strains.

1.3   INFLUENZA PREVENTION AND TREATMENT

Influenza virus is contagious, and spread of the disease can occur through direct contact with an infected individual, indirect contact with an inanimate object that carries germs from the infected individual, or through airborne transmission (coughing, sneezing) [2, 12]. Typical clinical symptoms of the pandemic 2009 A(H1N1) and seasonal A(H1N1) virus include fever, sneezing, coughing, headache, muscle aches, and sore throat, which make them hard to distinguish from one another [2, 8, 12]. The risk of influenza or influenza-associated complications is highest among children, the elderly, pregnant women, and people with other health conditions like heart disease or weakened immune systems [12].

1.3.1   Vaccines

The best protection against seasonal influenza is the vaccine, which can help prevent the spread of disease from person to person. The CDC recommends all eligible people receive the vaccine annually, and children 6 months to 8 years of age may receive two doses. Each year, the influenza vaccine is created to protect against three (trivalent) or four (quadrivalent) of the most prominent strains that are most likely to cause disease as predicted through surveillance [12]. The vaccine works by triggering an immune response in the body to produce antibodies and T

cells to fight off the infection [13]. After a person receives the vaccine, it takes approximately two weeks for protection to develop against influenza [12].

Influenza vaccines are time-consuming to make and require the expertise from people in many fields, including microbiology, epidemiology, and veterinary [13]. Each year, the World Health Organization consults with experts to decide which strains of influenza are likely to circulate the following year and should be placed in the vaccines. Vaccine strains are chosen using past viral strains, and therefore antigenic shift and drift are impossible to anticipate [2]. Influenza vaccines are produced by private manufacturers who are approved and licensed by the U.S. Food and Drug Administration (FDA). Manufacturers approved for the 2015-16 influenza season included GlaxoSmithKline, ID Biomedical Corporation of Quebec (distributed by GlaxoSmithKline), Sanofi Pasteur, bioCSL, Novartis Vaccines and Diagnostics, Protein Sciences, and MedImmune. Vaccines differ by production type, dose, and number of strains [12].

There are two types of vaccines available to protect against influenza, the Inactivated Influenza Vaccine (IIV) and the Live Attenuated Intranasal Vaccine (LAIV). IIV does not contain any live virus and is given through intramuscular injection with a needle. IIV viruses are inactivated by chemicals, such as beta-Propiolactone (BPL) or formaldehyde [14], or through inactivation with heat, ultraviolet light, or gamma rays [15]. LAIV is administered intranasally as a spray and contains a weakened live virus [12]. LAIV viruses are attenuated using heat; they cannot grow well in a host, but replicate enough to elicit an immune response. The vaccine virus is only able to cause infection in cooler temperatures, such as those found in the nose; they cannot infect warmer areas of the body such as the lungs [16]. The U.S. FDA approves private

sector manufacturers to make vaccines using various methods, including egg-based, cell-based, and recombinant influenza vaccines [16].

Traditionally, influenza vaccines are egg-based and viruses are grown in millions of fertilized chicken embryos. The virus is injected into the egg and allowed to incubate for several days so that the virus can multiply. The virus is harvested and put through a purification process where the manufacturer and FDA test the virus for potency and safety. Once approved, the different strains produced for that year are mixed together and standard doses are placed in containers including vials, syringes and nasal sprayers where it undergoes more quality control tests, such as for sterility. A sample of each lot and results are sent to the FDA, which confirms the results and allows the manufacturer to begin shipping the vaccines throughout the US. This process takes approximately 6 months from start to completion [13].

In 2010, the FDA pushed to find an easier and faster way to make influenza vaccines using cell culture technology, and by 2012 had approved cell-based influenza vaccines that use mammalian cell lines [13, 16]. Cell-based vaccines also begin with egg-grown virus. Instead of incubating the virus in eggs, it is cultured using Madin-Darby Canine Kidney (MDCK) cells and allowed to replicate. The virus is harvested from the MDCK cells, purified, and undergoes the same testing as egg-based vaccines [16]. The FDA believed that this advancement would help the US respond to public health emergencies in a timely manner. Advantages of this new cell technology include the ability to freeze the cells for later use, not relying on the availability of eggs for production, and potentially growing better viruses at a quicker rate [13].

The latest vaccine production type was approved in 2013. Unlike egg-based and cell-based vaccines, recombinant technology allows for the production of virus without using egg-grown virus or eggs in the production process. Instead, these "recombinant" vaccines use a

protein isolate, like HA, from the recommended circulating virus strain. The isolate is then combined with a virus that grows well in insect cells, mixed with those cells, and allowed to replicate. The influenza protein isolate (HA) is then harvested from the insect cells and purified before placement in the egg-free vaccine. This method is the only FDA approved egg-free method, and currently only one vaccine is available in the U.S. It is the quickest method because it does not depend on an egg supply [16].

Influenza viruses are constantly evolving, utilizing antigenic shift and drift, which is why a new vaccine is required every year. Scientists around the globe are constantly working to collect influenza samples to try to identify which strains will most likely be circulating during the next influenza season. One inherent problem with influenza vaccines is the need for the FDA to make its recommendation on which strains should go into the vaccine for the upcoming influenza season each year. The strains must be decided well in advance of the influenza season so that various manufacturers using different technologies can begin to make the vaccine for the fall [13]. Since the current influenza season has not ended when strain selection occurs, it is possible to choose the incorrect strains to be placed in the following year's vaccine. IIV and LAIV have both been proven to be effective in children and adults, and for the 2015-16 season the Advisory Committee on Immunization Practices does not recommended one over the other. However, there are certain groups of people who are recommended to not receive the LAIV, including those aged <2 or >49 years, pregnant women, immunocompromised individuals, and individuals with other certain health conditions. Efficacy of the vaccine depends on the characteristics of the person receiving the vaccine (age and current health-status) and whether the selected vaccine strains are a good fit for what is circulating that year. For the 2014-15 influenza season, laboratory analysis determined that the selected influenza A strains were antigenically or

genetically different from the strains that were circulating in the environment, resulting in reduced effectiveness of the vaccine [16]. When this occurs, it is pertinent to have good antiviral drugs available to help reduce the effects of an infection.

1.3.2 Antivirals

When the influenza vaccine fails, the community must then rely on antiviral drugs to help combat influenza infection. Antiviral therapy is recommended for those with severe disease or who are at-risk of contracting severe disease, including the elderly, pregnant women, and people with autoimmune disorders [2]. Researchers use the virus life cycle to study influenza and determine new ways to block infection. Currently, there are two classes of antivirals on the market to protect against influenza, matrix 2 (M2) channel inhibitors and neuraminidase (NA) inhibitors [3, 5].

There are two M2 ion channel blockers on the market, amantadine (Symmetrel™, Endo Pharmaceuticals) and rimantadine (Flumadine™, Forest Pharmaceuticals). M2 ion channel proteins are transmembrane proteins that form proton ion channels across the viral membrane. These proteins transport protons from the late endosome into the interior of the virus. Acidification resulting from this transfer induces a hemagglutinin conformation change so that the virus can fuse and infect host cells by releasing the viral RNA into the cytoplasm. M2 ion channel blockers bind the interior of the ion channel, blocking proton transport, and preventing the hemagglutinin conformation change that allows membrane fusion [17]. Adamantane antivirals only protect against influenza A. The FDA does not typically recommend these drugs because there have been high levels of antiviral resistance in the last few influenza seasons, while resistance against oseltamivir, zanamivir, and peramivir is still low [10, 12].

In the US during the 2014-2015 influenza season, there were three FDA-approved NA inhibitors available to treat or prevent disease from influenza A and B viruses: oseltamivir (Tamiflu™, Roche), zanamivir (Relenza™, GlaxoSmithKline), and peramivir (Rapivab™, BioCryst) [10, 17]. NA is a surface protein on the viral envelope that consists of four identical subunits. Its main role is to assist with viral progeny release from the infected cells. This is achieved by cleaving the glycosidic bond between the other major glycoprotein, hemagglutinin, and the terminal sialic acid receptors of the host cell. NA also assists with the break down of mucus in the respiratory tract, making it easier for the virus to attach to respiratory epithelium [18]. NA inhibitor antiviral drugs target the NA enzyme active site, located in the center of each subunit, which contain a number of highly conserved amino acid residues [19]. These drugs reduce infection by competing for NA binding and preventing progeny release of the virus from infected cells. Oseltamivir and zanamivir remain the most widely-used treatment against influenzas A and B and can reduce the duration of symptoms from about 1 to 3 days if administered within 48 hours of symptom onset [2].

Prospective future recommended treatments include laninamivir, a newer NA inhibitor, and favivirapir, a nucleoside mimic that inhibits viral RNA-dependent RNA polymerase, that can inhibit common oseltamivir-resistant strains of influenza virus. Fludase acts like NA, but destroys the host cell sialic acid receptors, leaving the virus with nothing to bind. There are also experimental drugs aimed at blocking the hemagglutinin protein, including an Entry Blocker (EB) peptide that binds HA to exhibit broad-spectrum antiviral activity, and peptide NDFRSKT, which was discovered using an antiviral screening method [20].

One of the most popular prescription medications for treating influenza types A and B is oseltamivir, or Tamiflu. Administration of Tamiflu through oral suspension is 6 mg/mL. If

infection occurs, treatment should be taken twice daily for 5 days; if being used as a prophylaxis due to a community outbreak, treatment is once daily for 10 days up to 6 weeks [21]. The treatment must be used close to the time of infection in order for it to be effective [22]. Drug-resistant mutations have emerged as variants of NA that bind and block oseltamivir from the active site. Resistance to oseltamivir has spread from the pre-2009 pandemic H1N1 virus to encompass the 2009 pandemic virus [22]. During the U.S. 2013-14 influenza season, researchers from the Influenza Antiviral Working Group found that 59/4,968 (1.2%) of the predominant pandemic 2009 virus had the H275Y oseltamivir mutation that aids in antiviral resistance by decreasing NA stability [3, 23]. Only 15 of 49 (30.6%) of those patients with a resistant virus infection had received oseltamivir prior to sample collection. Pennsylvania was one of the top states to provide oseltamivir-resistant virus specimens, 0/14 of which had exposure to oseltamivir prior to sample collection. Before 2007, the number of NA-inhibitor-resistant viruses was low (<1%) due to fitness costs, but emergence of oseltamivir-resistant seasonal influenza A(H1N1) H275Y viruses began in the 2007-08 influenza season, and by 2008-09 were as high as 100% in some countries [3, 23]. This sharp increase is most likely due to an evolutionary advantage of H275Y variants, and is not attributed to oseltamivir use. Researchers fear that the H275Y substitution may become fixed in the influenza A(H1N1)pdm09 viral genome as it did in the seasonal virus [23]. To counter this resistance, we need to develop new broad-spectrum treatments that will not result in virus resistance.

Designing drugs that are less susceptible to antiviral resistance will be challenging and will depend upon highly integrated experimental and theoretical studies. This field is likely to shift toward high-throughput screening that can help identify potential resistance-conferring mutations. This selection process includes using random mutagenesis of codons or nucleotides

from more conserved genes like NA and HA in plasmids to create mutant libraries that are passaged through tissue culture with and without a drug. Sequencing of each variant takes place before and after selection to determine the relative fitness. Among its many significant findings, this process has revealed that HA has a high mutational tolerance [3].

1.4 EXPERIMENTAL ANTIVIRAL PEPTIDE HB36.6

Our collaborates from the University of Washington have developed a novel small 94 amino acid protein, HB36.6, which is designed for use as a pre-exposure prophylactic and post-exposure therapeutic against influenza virus. Dr. David Baker created the peptide using his innovative computational modeling program (Rosetta). He created the peptide to interfere with influenza infection by binding the highly conserved HA stem region, therefore neutralizing a wide range of influenza variants. The peptide was optimized using deep mutational scanning, and was designed to interact similarly with seasonal, pandemic, and antiviral-resistant influenza strains. It protects against human group 1 strains including H1N1 and H5N1. The peptide has demonstrated a strong biopotency in vivo using intranasal delivery of the peptide in a mouse model and challenge with various group 1 influenza strains. This peptide acts as a hemagglutinin inhibitor by targeting a neutralizing epitope on HA and inhibiting fusion of the virus with the host cell [6].

In the mouse model, a prophylactic dose of the peptide will specifically block infection at the mucosal site of exposure, while administration post-infection reduces disease and accelerates viral clearance. The peptide binds directly to the virus without engaging a host receptor or invoking an immune response, and suppresses inflammatory (cytokine) responses associated

with influenza disease. This peptide has proven to bind with equal or higher affinity than some monoclonal antibodies (mAbs), which also bind the HA stem to inhibit the conformational changes required for membrane fusion. MAbs are a typical form of treatment in hospitals for critical influenza patients; they broadly bind and neutralize all group 1 influenza viruses in vitro. The new peptide does not protect against group 2 viruses because of the location of a glycan on the group 2 binding site that disrupts the binding pocket. However, unlike mAbs these proteins lack an Fc, the critical component for its broad protective effects in vivo through its interaction with the FcγR [6].

Drs. Baker and Fuller began to reevaluate HB36.5 by constructing a library and using random mutagenesis of its nucleotides for selection against seven different group 1 HA subtypes [24]. The libraries were sequenced and the Baker laboratory computed the enrichment or depletion that accompanied each individual point mutation during affinity maturation. A new library was then made consisting of the most optimal substitutions for all subtype selections at 12 mutated positions and carried out several rounds of yeast display sorting against A/South Carolina/1/1918 (H1N1) HA, which developed into a variant with nine substitutions (HB36.6). Using negative-stain electron microscopy they confirmed that HB36.6 binds to the designed target location on the HA (Figure 1A-C), and biolayer interferometry showed the new peptide had high affinity (Figure 1D) and comparable equilibrium binding constants (Figure 1E) against all tested HA subtypes. Compared to Ribavirin (a small molecule broad-spectrum antiviral approved in the US for treatment of Hepatitis C and Respiratory Syncytial Virus that neutralized influenza in vitro and protects against influenza virus in mice) and oseltamivir, HB36.6 displayed stronger in vitro antiviral potency. Co-treatments with oseltamivir and HB36.6 also

enhanced survival and lessened weight change in mice challenged with a lethal dose of influenza

virus better than either antiviral treatment alone [6].
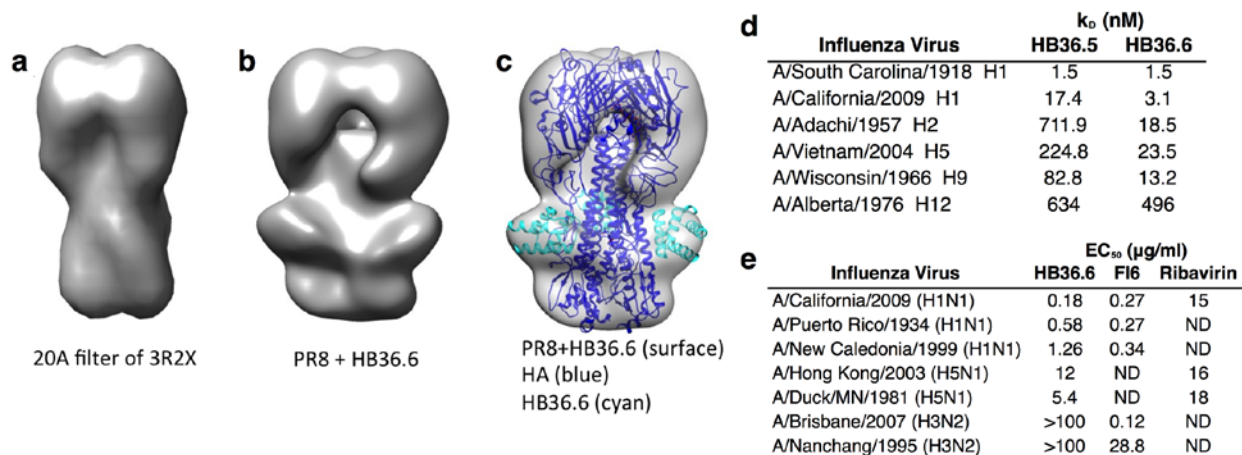
Figure 1. Characterization of HB36.6.

(**A**) HA protein of A/South Carolina/1/1918 (H1N1). (**B**) PR8 HA bound to HB36.6. (**C**) HA protein from A/South

Carolina/1/1918 (H1N1) (blue) with HB36.6 (cyan) docked into PR8 in B showing HB36.6 fits well into the stem

region. (**D**) Equilibrium binding constants for HB36.5 and HB36.6 against various HAs demonstrated broad binding

of group 1 subtypes. (**E**) EC_{50} (µg/ml) (compound concentration that reduces viral replication by 50%) of HB36.6,

the monoclonal antibody FI6v3, and ribavirin against group 1 and group 2 viruses. ND = not determined [6].

1.4.1 Preliminary Data

The Fuller laboratory has shown that HB36.6 can block infection in Balb/c mice when

administered prophylactically and reduces typical influenza symptoms while shortening disease

when administered therapeutically at the mucosal site of exposure *in vivo*. They found that when

mice were given a pre-exposure high dose of 6 mg/kg or low dose of 0.01-3 mg/kg HB36.6 at

≤48 hours before challenge with a lethal dose of mouse adapted H1N1 (A/California/07/2009),

the treated mice showed 100% survival and less weight loss compared to untreated controls,

which all died within 4-9 days post-infection (Figure 2A-B). The afforded protection by HB36.6

was dependent on the intranasal (IN) route of antiviral delivery because the same antiviral dose administered intravenously (IV) was not protective. HB36.6 was tested against other strains of influenza to see if results differed by strain (Figure 2C); results were similar amongst different strains. To evaluate HB36.6 as a therapeutic, mice were treated IN after influenza exposure using either a single dose of 3 mg/kg HB36.6 given on day 0 (2 hours post infection), +1, +2, or +3 days PI, or the same dose administered daily on days +1-4 PI (Figure 2D). When administered only once within 24 hours of infection, HB36.6 afforded a 60% survival rate, while administration daily after exposure afforded mice 100% survival with complete recovery in pre-exposure weights [6].
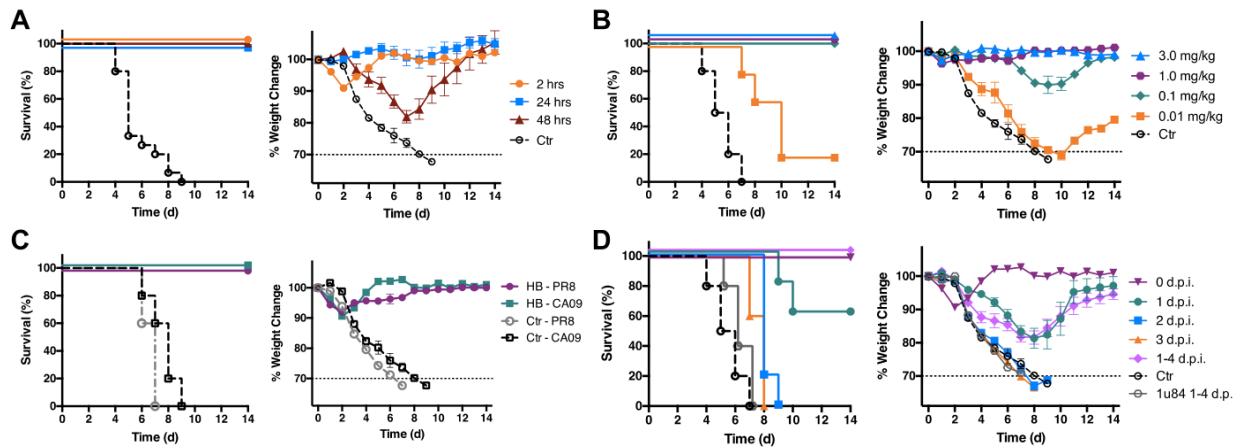
Figure 2. HB36.6 protects against challenge with lethal dose of influenza virus in mice.

Survival and weight change in (**A**) BALB/c mice treated with 6 mg/kg of HB36.6 at 2, 24, or 48 hours before challenge with 10 MLD_{50} CA09 H1N1 virus; (**B**) BALB/c mice treated with 0.01–3 mg/kg doses of HB36.6 2 hours before challenge with 10 MLD_{50} of CA09 virus; (**C**) BALB/c mice treated with 3 mg/kg of HB36.6 2 hours before infection with 10 MLD_{50} of CA09 virus or 6 MLD_{50} A/PR/8/34 (PR8) and (**D**) BALB/c mice treated with 3 mg/kg of HB36.6 on day 0 (2 hours post-infection) or +1, +2, or +3 days post-infection (d.p.i.), or once daily with HB36.6 or control protein (sham, 1u84) on days +1-4 post-infection with 10 MLD_{50} CA09 virus. Graphs are shown with mean with SEM [6].

HB36.6-treated mice showed lower viral titers in nasal secretions and lung tissues on days 2, 4, and 6 PI (Figure 3A-B). HB36.6 treatment (6 mg/kg) 24 hours before or 24 hours after lethal influenza challenge greatly suppressed the amounts of influenza-induced inflammatory cytokines (including IL-6, IL-10, IFN-g and TNF-α) detected in tissues at day 2 PI (Figure 3C). Antiviral cytokines were not detected in mouse lungs collected 24 hours after IN administration of HB36.6. This shows that protection provided by HB36.6 is not linked to the induction of antiviral cytokines in the lungs. After four weeks of treating mice biweekly with HB36.6, they were challenged with H1N1 to test the binder's biopotency. The binder did not induce antibodies in the weeks prior to influenza challenge and still provided 100% protection from mortality. Protection was specific to HB36.6 binding to the HA because daily administration of the control scaffold protein (1u84) that HB36.6 is modeled on provided no protection [6].
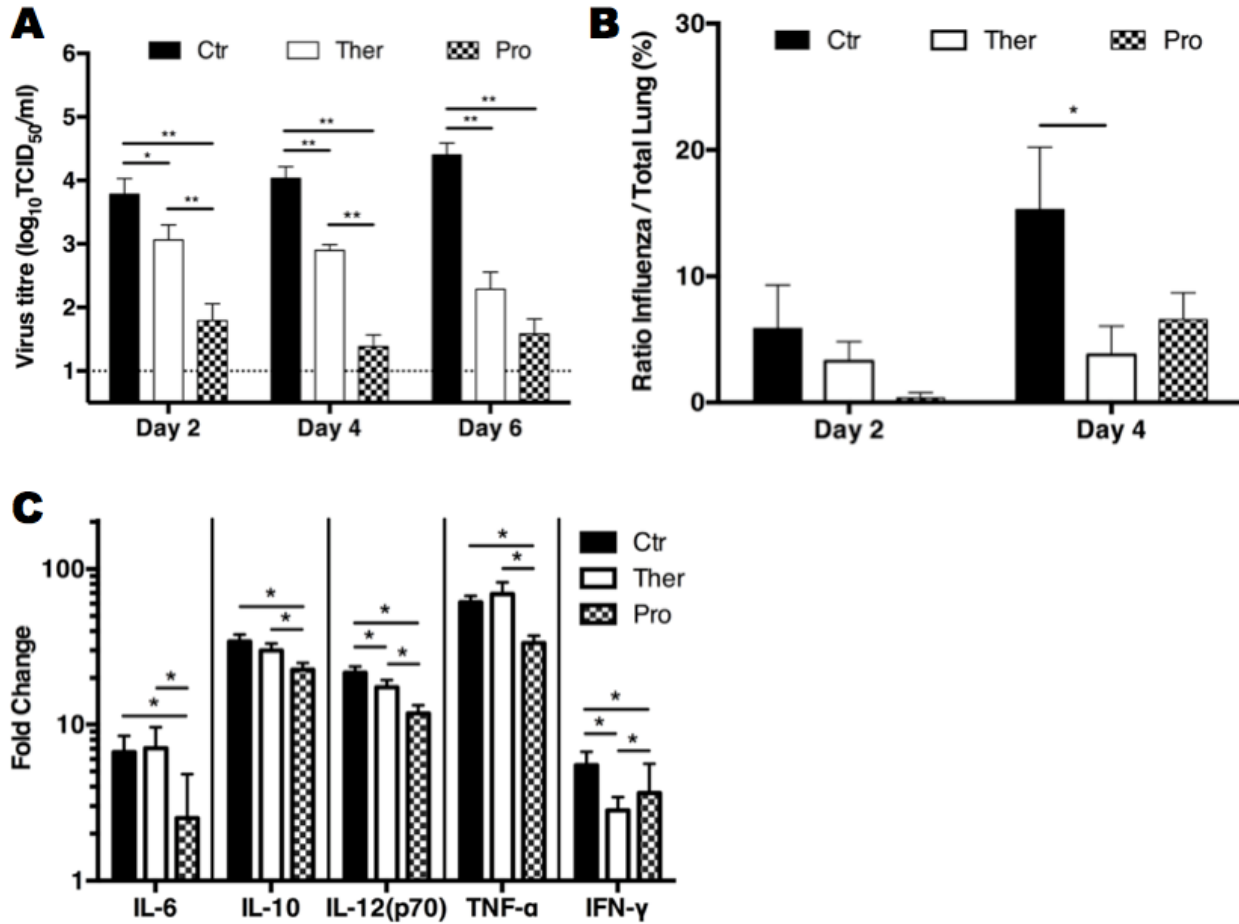
Figure 3. HB36.6 reduces viral replication and inflammation.

(**A**) Nasal wash viral titers of untreated infected controls (Ctr) and mice that were treated with 6.0 mg/kg HB36.6 24 hours before (Prophylaxis, Pro) or 24 hours after (Therapeutic, Ther) infection with 10 MLD_{50} CA09 influenza virus. (**B**) Quantification of influenza positive cells in lung tissues performed by measuring the area of positive staining compared to the total tissue on the slide. (**C**) Inflammatory cytokines from lung homogenates obtained from BALB/c mice on day 2 post infection shown using the fold change over naïve-uninfected mice. Significant differences between the Pro and Ther groups to the Ctr group are shown: *$P < 0.05$, **$P < 0.001$ [6].

HB36.6 shows promise as an effective antiviral in the mouse model. Our lab has collaborated with the Fuller lab to further evaluate HB36.6 in the ferret model, a model that can afford evaluation of the peptide against influenza viruses without adaptation and that exhibits a disease course that more closely mimics humans. Ultimately, the goal of these studies is to

broaden the antiviral efficiency to include protection from group 2 subtypes by combining a second small protein to work along with HB36.6. This would allow the antiviral to protect from up to 16 different subtypes of influenza as well as drifted and shifted genetic variants of the virus. Remaining uncertainty about host-pathogen interactions, vaccination, and lack of more efficient treatments limits our knowledge of influenza viruses [15]. These current studies seek to evaluate one new promising therapeutic candidate that will help to move the field closer to understanding these interactions, and hopefully identify a treatment that can move forward into humans.

2.0   STATEMENT OF THE PROJECT AND SPECIFIC AIMS

Approximately 5-20% of the US population gets sick with influenza annually, and more than 200,000 people are hospitalized due to influenza-related complications [12]. Currently, the best protection against seasonal influenza is vaccination, but the correct strains are difficult to predict, and when predicted incorrectly, are less effective. There are some prescription medications available to treat influenza, including oseltamivir (Tamiflu), a neuraminidase inhibitor, which was approved by the US Food and Drug Administration in 1999. However, Tamiflu has received mixed reviews in its effectiveness to treat seasonal influenza [9]. Given the incident rate of influenza A, and the increasing number of elderly and immune compromised people around the globe, it is crucial to develop more effective ways of protecting these more vulnerable populations.

This project is a combination of efforts from laboratories at the University of Washington and University of Pittsburgh, which seeks to test an antiviral drug, HB36.6, to protect against influenza A infection. HB36.6 is a small protein that interferes with influenza infection by binding the virus' hemagglutinin surface protein. This peptide has proven to bind with equal or higher affinity than monoclonal antibodies, which are used as a form of treatment in hospitals for critical influenza patients. Previous studies have determined HB36.6's efficiency in the mouse model. Mice are widely used in influenza studies, but they are not a natural host for influenza virus infection and cannot be naturally infected with human strains like ferrets [1, 11]. The ferret

was first discovered to be susceptible to human influenza in the 1930s and has since been widely used as a model for the disease [5]. The ferret is an appropriate model for influenza infection because it displays clinical signs of disease similarly to humans in that it produces fever, sneezing, generates nasal discharge, and exhibits lowered activity levels [11]. Our lab has now tested the antiviral's effectiveness using the highly pathogenic influenza A H1N1 strain inside a ferret model. These studies have yielded important information on a new class of influenza therapeutics that can be further evaluated in humans. **The overall hypothesis for this study was that HB36.6 would be effective in reducing viral loads and limiting disease following aerosolized influenza infection in the ferret model, and this reduction in viral loads/disease would be more effective compared to the currently used antiviral, Tamiflu.**

2.1 AIM 1: TO DETERMINE WHETHER TREATMENT WITH HB36.6 RESULTS IN REDUCED CLINICAL SIGNS OF DISEASE COMPARED TO UNTREATED CONTROLS FOLLOWING AEROSOLIZED INFLUENZA INFECTION IN THE FERRET MODEL

The ferrets were monitored and scored using a detailed clinical scoring system developed in collaboration with Dr. Fuller for onset of clinical signs of disease. They were also tracked through daily measurements of weights and temperatures. From these observations, we have determined the differences in physical indications of disease between each group of ferrets. This aim was important in assessing the effectiveness of HB36.6. From these observations, results demonstrated less severe clinical disease in ferrets treated with HB36.6 compared to untreated controls.

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2.2 AIM 2: TO DETERMINE WHETHER TREATMENT WITH HB36.6 REDUCES VIRAL TITERS IN NASAL WASH AND LUNG TISSUE SAMPLES COMPARED TO UNTREATED CONTROLS FOLLOWING AEROSOLIZED INFLUENZA INFECTION IN THE FERRET MODEL

Viral titers were determined through viral RNA extraction of nasal swab samples, followed by one-step quantitative real-time reverse-transcription PCR to evaluate samples collected at day 0 (pre-infection) through day 3 or 6-7 (necropsy took place on day 3 for study #2; day 6 for study #3; and day 6 or 7 for study #4). Viral titers in lung samples were determined after viral RNA extraction followed by one-step quantitative real-time reverse-transcription PCR using tissues harvested at necropsy. In many viral infections, viral titer is associated with severity of disease. However, in influenza infection this is not always so clear. It has been hypothesized that the immune response to influenza infection, often referred to as the "cytokine storm", may contribute as much, if not more, to the pathogenesis of disease in influenza infection [25, 26].

We further attempted to measure innate immune responses as reflected by cytokine responses in serum, nasal wash, and lung tissue samples. Determination of the specific types and quantitation of each cytokine response produced in ferret samples after influenza infection was attempted using total RNA extraction followed by RT-PCR. We hypothesized that If HB36.6 was successful in limiting disease, it would likely suppress or alter the profile of cytokines released in response to infection. A comparison of cytokine RT-PCR in influenza infected vs. control tissues would provide insight into cytokines that may be suppressed or enhanced in response to infection.

This project has tested the efficiency of a novel antiviral protein, HB36.6, to protect against influenza A. We anticipated that HB36.6 would be effective at reducing viral loads and limiting disease compared to untreated controls, and believed HB36.6 would be more effective than Tamiflu in the ferret model. This new class of antivirals has the potential to be further evaluated in humans, and once further expanded, can be used as a prophylactic or therapeutic treatment for many types of influenza.

3.0   MATERIALS AND METHODS

3.1   BIOSAFETY

All experiments using live influenza A H1N1 virus were performed in a biosafety level 2 (BSL-2) laboratory in the Center for Vaccine Research at the University of Pittsburgh. Protective gowns and latex gloves were worn when working with live virus under a class II biosafety cabinet (BSCII).   The cabinet was sterilized using Vesphene® IIse (STERIS Corporation, cat. #6461-08) diluted 1:128 with water, and 70% ethanol as disinfectants.   All personnel were trained in sterile technique as well as working with infectious virus prior to being able to work independently with influenza virus.   The laboratory was inspected and monitored at least annually by the Department of Environmental Health & Safety.

3.2   VIRUS PROPAGATION

A stock of 2009 H1N1 influenza virus A/California/07/2009 (2.6×10^8 $CEID_{50}$/ml obtained from BEI Resources, cat. # NR-13663) was used to infect white leghorn fertile specific pathogen-free chicken eggs obtained from Charles River (North Franklin, CT) using the procedure described in Appendix A.   A high virus titer of 2.6×10^7 $TCID_{50}$/ml was confirmed using $TCID_{50}$ to measure cytopathic effect in MDCK cells.

3.3   AEROSOL INOCULATION

Aerosol exposure containing harvested influenza H1N1 virus was performed using a 3-jet Collison nebulizer (BGI, Inc. Waltham, MA).   The nebulizer is located inside a class III biological safety cabinet (Baker Co., Sanford, ME) maintained under negative pressure, and is controlled through the AeroMP bioaerosol exposure system (Biaera Technologies, Hagerstown, MD) [27]. It creates a uniform particle aerosol between 1-2 μm in size, which deposits in the deep lung. The ferrets were placed in wire-mesh cages, one ferret per cage, and transported into the regional biocontainment laboratory (RBL) to the aerosol suite for exposure inside a whole-body aerosol inhalation chamber (Biaera Technologies). All ferrets were exposed to the aerosol for 20-30 minutes (20 min for study #2 and 30 min for studies #3 and #4) followed by a 5-minute air wash, after which they were returned to the animal holding facility.

Aerosol concentration of the virus was determined through constant sampling of the chamber using an all-glass impinger (AGI; Ace Glass, Vineland, NJ) containing 10 ml viral growth media. Presented dose was calculated using Guyton's formula ($V_m = 2.10 \pm W_b^{0.75}$, where W_b is body weight) for respiratory minute volume (V_m) [28], and multiplying the total volume (V_t) of experimental atmosphere inhaled ($V_t = V_m$ x length of exposure) by the aerosol concentration (C_e) (presented dose $= C_e$ x V_t) [29].

For Fuller Ferret Study #2 exposure took place in 3 runs of 4 ferrets for 20 minutes. The nebulizer concentration was 1.65 x 10^5 $TCID_{50}$ with a spray factor (SF) of 7.56 x 10^{-7} (runs #1 and #3) and 1.11 x 10^{-6} $TCID_{50}$ (run #2). The average presented dose was estimated to be 879 $TCID_{50}$ of pH1N1. For study #3 exposure took place in 3 runs of 3 ferrets for 30 minutes. The nebulizer concentration was 1.47 x 10^5 $TCID_{50}$ with a SF of 1.2 x 10^{-6} (runs #1 and #3) and 1.6 x

10^{-6} TCID$_{50}$ (run #2). The average presented dose was estimated to be 1,711 TCID$_{50}$ of pH1N1. For study #4, exposure took place in 4 runs of 3, 3, 4, and 3 ferrets (total 13 ferrets) for 30 minutes. The nebulizer concentration was 4.22 x 10^6 TCID$_{50}$ with a SF of 5.56 x 10^{-8} (run #1), 8.98 x 10^{-8} (runs #2 and #3), and 2.36 x 10^{-7} TCID$_{50}$ (run #4). The average presented dose was estimated to be 4,818 TCID$_{50}$ of pH1N1. Individual inhaled dose per ferret for all studies can be seen in Appendix B.

3.4   FERRET STUDIES

For each study, H1N1 sero-negative ferrets were received through the Division of Laboratory Animal Resources (DLAR) from Triple F Farms (Sayre, PA). Ferrets were housed and necropsied in the animal holding and procedure room of a BSL-2 facility, and were under the watchful care of DLAR technicians for the duration of the study. The University of Pittsburgh abides by all federal regulations governing the use of animals in research in the U.S. and is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited institution. All procedures were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (IACUC). Ferrets were subcutaneously microchipped using IPTT-300 implantable electronic ID transponders (BioMedic Data Systems, Inc., Seaford, DE) after arrival and allowed to acclimate before aerosol infection with A/California/07/09 pandemic H1N1 (pH1N1) influenza virus.

Each ferret weight, temperature, and clinical score was recorded daily starting before infection at day 0 through necropsy. Ferrets were monitored using clinical assessment sheets developed by Dr. Deborah Fuller's laboratory (University of Washington) in collaboration with

the Cole lab. This assessment encompasses the categories of appearance, movement, pulmonary function, and consumption/elimination (shown in Appendix A). Clinical assessments were performed and recorded by both DLAR technicians and Cole lab members. In the event that ferrets displayed severe signs of disease, the DLAR veterinarian was consulted. The study design called for any ferret that lost ≥30% of their total body weight or that reached a clinical score of 10 in two or more of the clinical scoring areas and lost ≥20% of their body weight to be humanely sacrificed.

Ferret samples were collected for evaluation of viral, immunologic and pathologic evaluation of influenza infection. For all ferret samples, ferrets were sedated with 4% isoflurane by canine masking. Sedation was confirmed by toe pinch and slow steady breathing. Once sedated, nasal washes were collected daily for all studies; blood was collected daily for study #2, and on day 0 and necropsy for studies #3 and #4. Tissue and BAL were harvested at necropsy and were either flash frozen on dry ice until storage at -80°C or stored in 10% formalin (Fisher Scientific, cat. #SF984). The day following necropsy, the tissues in formalin were moved to clean tubes containing fresh formalin for eventual removal from the lab for pathology. Ferrets were held until they displayed signs of awakening from anesthesia, then were returned to their cages. Ferrets were monitored until they were fully awake and moving freely about their cages.

3.4.1 Study #2

Eleven ferrets, ages 16 to 18 weeks old and weighing between 0.70 and 0.86 kg, were received for this study. Three ferrets were given 10mg/kg of HB36.6 2 hours prior to pH1N1 challenge (medium-dose), four ferrets were given 2.5mg/kg of HB36.6 2 hours prior to pH1N1 infection (low dose), and four ferrets were used as untreated controls. All eleven ferrets were

aerosol challenged with pandemic H1N1, 2.6 x 10^7 A/California/07/09 TCID$_{50}$, and received an average inhaled dose of 879 TCID$_{50}$. The ferrets were monitored and sampled (nasal wash and blood) for 3 days following influenza infection, after which they were humanely sacrificed and necropsied on day 3 post infection.

3 Ferrets = 10mg/kg HB36.6 at -2hr
4 Ferrets = 2.5mg/kg HB36.6 at -2hr
4 Ferrets = untreated controls
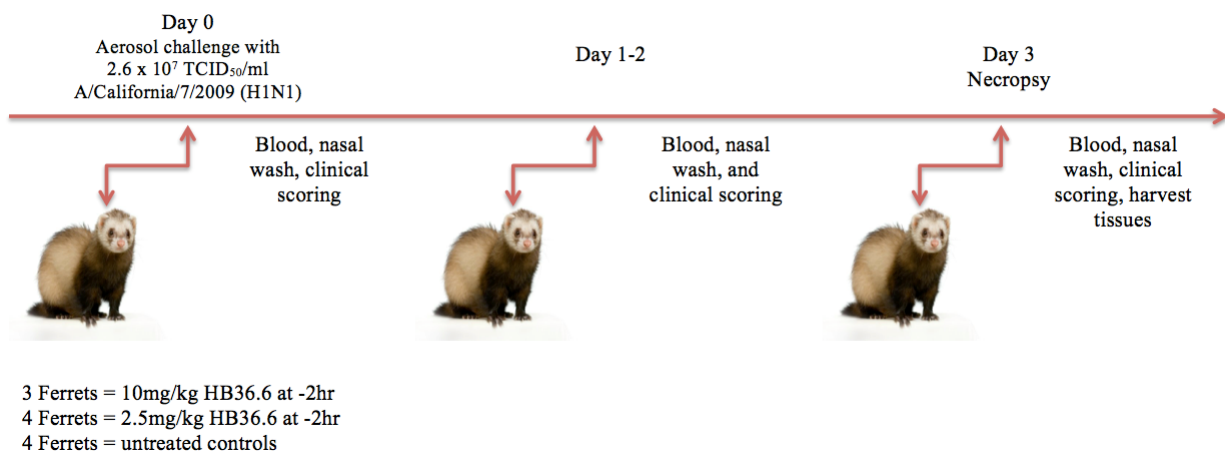
Figure 4. Study #2: Evaluate HB36.6 prophylactically in the ferret model using different doses of treatment compared to untreated control ferrets.

All ferrets were aerosol challenged with 2.6 x 10^7 TCID$_{50}$/ml of A/California/7/2009(H1N1) influenza virus. Two hours prior to infection, three ferrets were treated with 10mg/kg HB36.6 and four ferrets were treated with 2.5mg/kg HB36.6. Blood and nasal wash samples were collected daily, and clinical scoring was completed daily.

3.4.2 Study #3

Eleven ferrets, 16 to 18 weeks old and weighing between 0.67 and 0.81 kg, were received for this study. All ferrets were allowed to acclimate for 72hr after arrival before being microchipped. Two of the eleven ferrets were given 15mg/kg of HB36.6 2 hours before anticipated influenza challenge, but due to the limited volume of HB36.6 we were allowed to administer, we were unable to achieve the desired 20 mg/kg dose (IACUC protocol limited us to administration of ≤1cc per ferret at any one treatment period) these ferrets were pulled from

study #3 and not challenged. Instead they were allowed to rest and the antiviral (15mg/kg HB36.6) was given time to decay before use in study #4. The remaining 9 ferrets were used for study #3. Four ferrets were given 20mg/kg of HB36.6 binder 24 hours prior to pH1N1 challenge, two ferrets were given 20mg/kg of binder two hours prior to pH1N1 infection, and one ferret was given 10mg/kg of binder two hours pre-challenge with pH1N1 virus. Two ferrets were used as untreated controls. All nine ferrets were aerosol challenged with 2.6×10^7 $TCID_{50}$ A/California/07/09 pandemic H1N1 and received an average inhaled dose of 1,711 $TCID_{50}$. The ferrets were monitored and sampled (daily nasal washes) for 6 days following influenza infection, after which they were humanely sacrificed and necropsied on day 6 post infection.

Day 0
Aerosol challenge with
2.6×10^7 $TCID_{50}$/ml
A/California/7/2009 (H1N1)

Day 1-5

Day 6
Necropsy

Blood/serum and
nasal wash
collections,
clinical scoring

Clinical scoring
and nasal wash
collection

Blood/serum and
nasal wash
collections,
clinical scoring

4 Ferrets = 20mg/kg HB36.6 at -24hr
2 Ferrets = 20mg/kg HB36.6 at -2hr
2 Ferrets = untreated controls
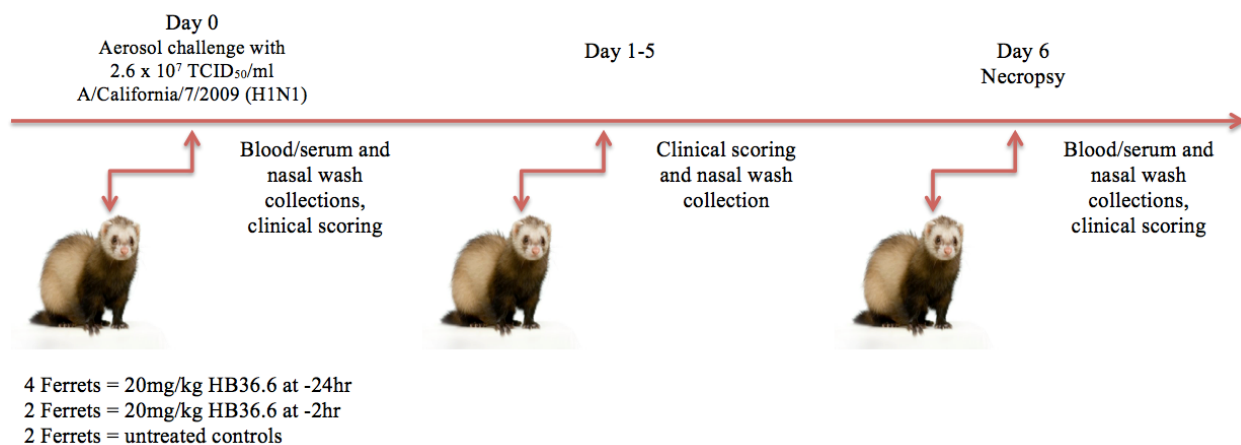
Figure 5. Study #3: Evaluate treatment with HB36.6 received at different prophylactic time points in the ferret model compared to untreated control ferrets.

All ferrets were aerosol challenged with 2.6×10^7 $TCID_{50}$/ml of A/California/7/2009 (H1N1) influenza virus. All treated ferrets received a single dose of 20mg/kg HB36.6; two ferrets were treated two hours before infection, and four ferrets were treated 24 hours before infection. Nasal wash samples were collected daily, and clinical scoring was completed daily. Blood samples were taken preinfection on day 0 and before necropsy on day 6.

3.4.3 Study #4

Eleven ferrets, 16 to 18 weeks old and weighing between 0.62 and 0.83 kg, were received for study #4. Two ferrets were given 20mg/kg of HB36.6 binder two hours prior to challenge with pH1N1, 4 ferrets were given 20mg/kg of binder once daily from days 1 to 5, and 4 ferrets were given 5mg/kg oseltamivir (Tamiflu) twice daily (morning and evening dose; dosing based on weight as directed). One ferret was used as an untreated control. All eleven ferrets were aerosol challenged with 2.6×10^7 TCID$_{50}$ A/California/07/09 pandemic H1N1 and received an average inhaled dose of 4,818 TCID$_{50}$. The ferrets were monitored and sampled (daily nasal washes) for 6 days following influenza infection, after which they were humanely sacrificed and necropsied on day 6 or 7 post infection.

2 Ferrets = 20mg/kg HB36.6 at -2hr
4 Ferrets = 20mg/kg HB36.6 once daily, days 1-5
4 Ferrets = 5mg/kg Tamiflu twice daily, day 1-5
1 Ferret = untreated control
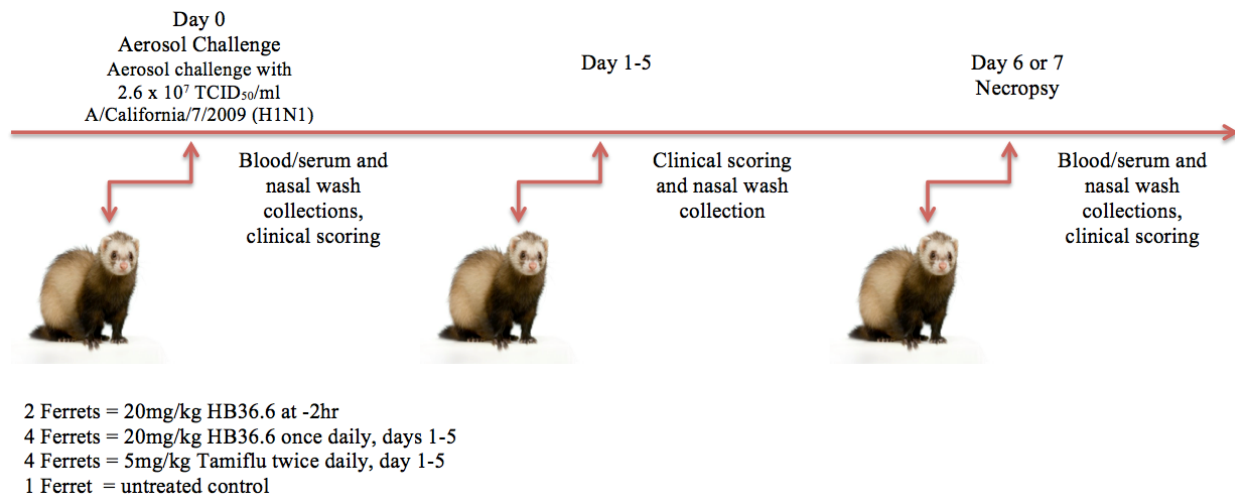
Figure 6. Study #4: Compare the efficacy of therapeutic HB36.6 treatment in ferrets to the currently used antiviral, Tamiflu.

All ferrets were aerosol challenged with 2.6×10^7 TCID$_{50}$/ml of A/California/7/2009 (H1N1) influenza virus. All HB36.6-treated ferrets received 20mg/kg HB36.6; two ferrets were treated two hours before infection, and four ferrets were treated daily on days 1-5 post infection. Four ferrets were treated with 5mg/kg Tamiflu twice daily on

days 1-5 post infection. Nasal wash samples were collected daily, and clinical scoring was completed daily. Blood samples were taken preinfection on day 0 and before necropsy on day 6 or 7.

3.1   BLOOD COLLECTION

Ferrets were isoflurane sedated by canine mask and blood was collected from the cranial vena cava using a 1cc syringe with a 25g safety needle. Blood was transferred to a 3cc heparin blood tube (Becton Dickinson), inverted several times and stored at room temperature until all samples were collected. Blood was taken to the lab, centrifuged at 1500rpm for 10 min to separate plasma from serum, and samples were stored at -80°C.

3.2   NASAL WASH COLLECTION

Nasal washes were performed with 1 ml (studies #2 and #3) or 2 ml (study #4) of sterile saline administered with an endotracheal tube on the end of a 3cc syringe. Saline was slowly dripped into the nares of isoflurane-sedated ferrets and then collected by gravity into sterile petri dishes. Ferret noses were additionally swabbed internally with a 6-inch sterile polyester-tipped applicator (Fisher Scientific, cat. #23-400-122). Samples were combined (polyester-tipped applicator in saline) and stored at -80°C. Real-time PCR was used to analyze samples after RNA extraction. To extract RNA from dry samples, PBS was used to wash the polyester-tipped applicator.

3.3   SAMPLES COLLECTED AT NECROPSY

At the end of each study, ferrets were humanely sacrificed. Ferrets were heavily sedated with 4% isoflurane, confirmed by toe pinch and slow, steady breathing. Nasal washes and final bleeds were performed as described above. Ferrets were then humanely sacrificed by injection of Beuthanasia at 5 mg/kg directly into the heart. Following injection, the absence of breathing and heart rate were confirmed before performing the necropsy.

For necropsy, the animals were moved to a sterile work space on the bench or inside a BSC. Bronchoalveolar lavage (BAL) was collected from all ferrets at necropsy using 10 ml sterile PBS administered into the lobes of the lungs via a feeding tube connected to a 10cc syringe. Once administered, the liquid was gently withdrawn, with approximately 50-60% recovery of liquid instilled. Following the BAL, the ferret was opened, and a gross pathological evaluation of organs was performed. Samples of most organs were obtained and either flash-frozen on dry ice/ethanol (nasal wash, BAL, trachea, 4 different lung lobes, nasal turbinate) or preserved in 10% paraformaldehyde (trachea, 2 lung lobes, nasal turbinate).   If indicated by disease course, a craniotomy was performed, and brain sections and spinal cord were also collected.

3.4   RNA EXTRACTION

Tissue sample RNA was isolated using a Qiagen RNeasy Mini Kit (Germantown, MD, cat. #74104) and RNase-free DNase Set (Germantown, MD, cat. #79254) for on-column DNase digestion and homogenates were used for PCR analysis. From each tissue, approximately

400mg was homogenized in DMEM with 2% FBS, and RNA was extracted using TRI Reagent® Solution (Ambion, Thermo Fisher Scientific, cat. #AM9738). Liquid sample RNA was extracted using an Invitrogen PureLink® Viral RNA/DNA Mini Kit (Carlsbad, CA, cat. #12280-050). After extraction, one-step quantitative real-time reverse-transcription PCR was performed using the SuperScript® III Platinum® One-Step qRT-PCR Kit with ROX (Invitrogen, cat. #11745-100) following the protocol in Appendix A. All samples were detected using reagents provided in the Swine Influenza A (H1N1) Real-Time RT-PCR Assay kit (BEI Resources, cat. #NR-15577). All samples from study #2 were detected using the Swine Influenza H1 (swH1) primers/probe set based on the detection of the H1N1 HA gene (nucleotide sequence listed in Appendix A). Nasal wash RNA from days 3 and 6 (studies #3 and #4) were also detected using the swH1 primers/probe set. Nasal Swab (Days 0, 1, 2, 4, and 5; study #3) and lung 4/6 RNA (study #3 and #4) were detected using the Universal Influenza A (InfA) primer/probe set based on the detection of the H1N1 matrix (M) gene [30].

PCR of standards accompanied the samples; the standards were ten-fold dilutions (10^{-1} through 10^{-6}) of the A/California/07/2009 (BEI Resources, cat. #NR-13663) pandemic H1N1 virus (original titer: 2.8×10^8 $CEID_{50}$/ml) made by Dr. Amy Hartman. A second set of standards had to be made when Dr. Hartman's stocks ran out. This new standard curve consisted of 10-fold dilutions (10^{-1} through 10^{-6}) of A/California/04/09 (gift provided by Dr. Stacy Schultz-Cherry, St. Jude Children's Research Hospital) pandemic H1N1 virus (original titer: $10^{8.25}$ $TCID_{50}$/ml). Known viral titers were used to calculate quantities for the standard curve and quantities were verified by PCR. See Appendix A for making a standard curve and quantities of the standard curves used. Standard curve cycle threshold (Ct) values began at cycle ~13 for both swH1 and InfA primer/probe sets and each dilution was ~4 cycle thresholds above the next.

3.5   QRT-PCR FOR VIRUS TITERS

To analyze samples, real-time RT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using the reagents and methods in Appendix A. PCR was designed to run for 40 cycles using the parameters listed in Appendix A and AH H1N1 (for Universal Influenza A primer/probe set) or SW Inf H1 (for Swine Influenza H1 primer/probe set) as the detector. When the standards and controls met the expected results, the unknown samples were considered positive for pH1N1 if the reaction growth curve crossed the threshold line within 37 cycles.

3.6   CYTOKINE DETECTION

3.6.1   ELISA

An enzyme-linked immunosorbent assay (ELISA) was used in an attempt to detect interferon (IFN)-γ in ferret serum and blood samples from study #2 following an adapted protocol developed from [11] (shown in Appendix A). Antibodies used include Monoclonal Anti-Ferret Interferon Gamma, Clones 4A4B7 and 1H1H12 (BEI Resources, cat. #NR-4492 and #NR-4493), and peroxidase conjugated Goat Anti-Ferret IgG IgA IgM Antibody (Rockland™ Immunochemicals Inc., Limerick, PA). Optical density was determined using a PowerWave XS (BioTek) and SpectraMax L plate reader at 450nm.

3.6.2 RNA Extraction and cDNA Synthesis

RNA isolation was performed on serum, blood, peripheral blood mononuclear cell (PBMC) and nasal wash samples using the RNeasy® Mini Kit (Qiagen, cat. #774104) with on-column DNase Digestion (cat. #79254), PureLink Viral RNA/DNA Kit (Invitrogen, cat. #12280-050), or QIAamp® Viral RNA Mini Kit (Qiagen, cat. #52904). Then first strand cDNA synthesis was performed with 5μg RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat. #K1642). Primer sequences for ferret IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-10, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Integrated DNA Technologies, Coralville, IA) can be found in Appendix A.

3.6.3 Cytokine PCR

Two-step quantitative real-time RT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, cat. # K0221) with ≤500 ng Template DNA (not to exceed 10% of the total PCR volume). Analysis was done using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) set to the parameters listed in Appendix A. Upon successful detection of cytokines using PCR, expression levels should be normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and calculated as fold change compared to day 0 ferrets based on the ΔΔCt method. The change in threshold cycle (ΔCt) is calculated by subtracting the gene of interest at each time point from the GAPDH Ct value. Then fold change is calculated by subtracting the day 0 ΔCt values from the subsequent days ΔCt values. The formula: fold change $= 2^{-\Delta\Delta Ct}$ is then used to obtain absolute values [31].

4.0   RESULTS

The goal of these studies was to evaluate a novel antiviral, HB36.6, for its ability to inhibit infection of aerosolized influenza in the ferret model. To accomplish this, three animal studies were performed which evaluated dose and timing of treatment pre-infection as well as a therapeutic treatment regimen, including the standard of care Tamiflu, after aerosol exposure to pH1N1. Fuller Ferret Study #2 was a three-day low-dose (2.5mg/kg or 10mg/kg) prophylactic study with antiviral protein HB36.6. Study #3 was a six-day high-dose prophylactic study with 20mg/kg HB36.6. Study #4 was a six-day high-dose (20mg/kg) therapeutic study compared to the currently marketed antiviral, Tamiflu (5mg/kg as recommended). All ferrets were challenged with A/California/07/09 pandemic H1N1 and assessed daily using a detailed clinical scoring system. Blood, nasal wash, and tissue samples were harvested for virologic, immunologic and pathologic analyses to compare treated and untreated ferrets following aerosolized influenza infection.

Ferrets were prescreened for influenza negative status prior to selection for studies. For these analyses, hemagglutinin inhibition (HAI) assays were performed on the ferret sera to demonstrate lack of antiviral H1N1 antibodies prior to delivery to the University of Pittsburgh. HAI assays for study #2 were performed by Dr. Amy Hartman using ferret serum received 1-2 weeks before beginning the study; HAI assays for studies #3 and #4 were performed by the Cole lab.

Based on confounding results obtained with study #4, (mainly that two of the three untreated controls were minimally infected post challenge), HAI assays were also performed on serum collected at day 0 and necropsy to determine whether these ferrets had antibodies for influenza, demonstrating that they had been exposed/infected with influenza at the University of Pittsburgh prior to initiation of study #4. Study #4 control ferrets F15-010 and F15-011 had positive antibody responses in the HAI assay, explaining the low level of virus infection post challenge.

4.1 AIM 1: TO DETERMINE WHETHER TREATMENT WITH HB36.6 RESULTS IN REDUCED CLINICAL SIGNS OF DISEASE COMPARED TO UNTREATED CONTROLS FOLLOWING AEROSOLIZED INFLUENZA INFECTION IN THE FERRET MODEL

Observations for clinical signs of disease are an indicator of disease severity. Ferrets were assessed each morning for clinical signs of disease that included measurement of weights, temperatures, and observations that were recorded using a detailed clinical scoring sheet (shown in Appendix A). Temperature (°C) is shown for each group throughout the duration of the study in the form of a line graph. Weight information is displayed as percentage change compared to day 0 measurements in the form of a line graph. Clinical score data is displayed as a dot plot. A clinical score of 0 indicates no symptoms or normal behavior, and increased numbers correlate with increased signs of disease. All scores were adjusted to reflect additional written notes recorded by either the investigator or DLAR team that were not part of any category on the

clinical assessment sheet. Formal statistics were only completed for study #2, where the sample size was 3 or 4 for each experimental group.

4.1.1 Study #2

Study #2 evaluated low dose HB36.6 treatment in ferrets aerosolized with influenza. Elevations in temperatures were observed in all experimental groups, peaking at day 2 post infection (PI) and either returning to preinfection baseline or plateauing on day 3 PI (Figure 7A). While treated ferrets (2.5mg/kg and 10mg/kg) displayed greater weight loss over time compared to untreated control ferrets, these differences did not achieve significance (Figure 7B). For example, control ferrets experienced approximately 2% weight loss, while treated ferrets averaged approximately 5% weight loss. The untreated control group displayed significantly more severe clinical signs of disease (reduced food/water intake, increased nasal discharge, lethargy) by days 2 and 3 than the treated ferrets (Figure 7C).

A.

B.

ange (%)

C.
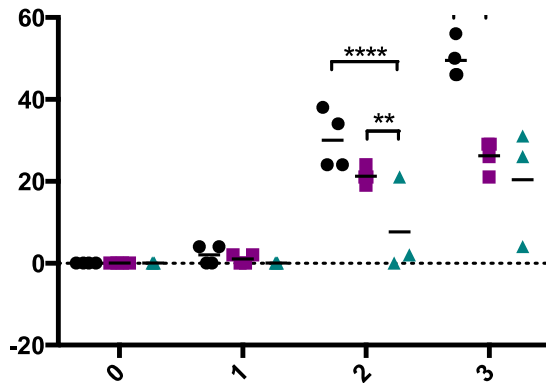
Figure 7. Clinical signs of disease in ferrets treated with low doses of HB36.6 and untreated control ferrets after aerosol exposure to A/California/07/09 (H1N1).

Study #2 consisted of four untreated control ferrets (black circles), four ferrets treated with 2.5mg/kg HB36.6 two hours before infection with pH1N1 (purple squares), and three ferrets treated with 10mg/kg HB36.6 two hours before infection (teal triangles). Ferrets were challenged with 2.6 x 10^7 $TCID_{50}$ A/California/07/09.   (A) Temperature (°C) of treated and untreated control ferrets over the course of infection. (B) Percentage of weight change of treated and untreated control ferrets over the course of infection. (C) Individual ferret clinical scores for treated and untreated control ferrets over the course of infection. Graphs A and B are shown with mean and SD at each time point. Graph C is shown with mean at each time point. Statistical analysis was done using a two-way ANOVA for multiple comparisons where $\alpha = 0.05$.

4.1.2   Study #3

Study #3 evaluated high dose (20mg/kg) HB36.6 treatment prior to influenza infection. For study #3, no notable changes in temperature were observed between treated and untreated groups (Figure 8A). All groups experienced weight loss. The untreated control group and ferrets treated with 20mg/kg at 24 hours before infection (−24hr) experienced the highest percentage in weight loss, demonstrating maximum average weight loss (15-20%) on day 3 PI (Figure 8B). This was in marked contrast to ferrets treated with 20mg/kg two hours before infection (−2hr), with only ~9% weight loss on day 3 and remaining steady up until ~11% weight loss on day 6 PI. On days 4 through 6 PI, untreated control ferrets displayed more severe clinical signs of disease than the treated groups, with -24h ferrets (treated with 20mg/kg HB36.6 at −24hr) displaying the fewest symptoms overall (Figure 8C). It was not possible to evaluate statistical significance due to the small sample size of untreated control ferrets and ferrets treated with 20mg/kg HB36.6 at −2hr (N=2).

A.

B.

Weight Change (%)

ost Infection

C.
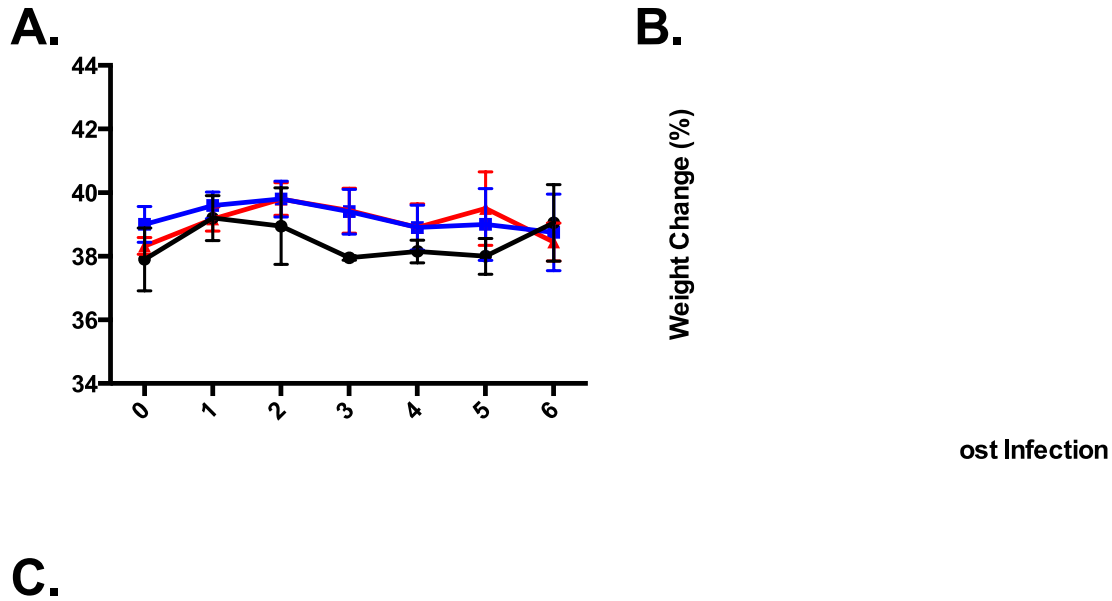
Figure 8. Clinical signs of disease in ferrets treated with a high dose of HB36.6 and untreated control ferrets after aerosol exposure to A/California/07/09.

Study #3 consisted of two untreated control ferrets (black circles), two ferrets treated with 20mg/kg HB36.6 two hours before infection with H1N1 (blue squares), and four ferrets treated with 20mg/kg HB36.6 24 hours before infection (red triangles). Ferrets were challenged with 2.6 x 10^7 $TCID_{50}$ A/California/07/09. (A) Temperature (°C) of treated and untreated control ferrets over the course of infection. (B) Percentage of weight change of treated and untreated control ferrets over the course of infection. (C) Clinical scores of treated and untreated control ferrets over the course of infection. Graphs A and B are shown with mean and SD at each time point. Graph C is shown with mean at each time point.

4.1.3 Study #4

Study #4 was designed to evaluate the therapeutic potential of HB36.6 in comparison to the standard of care, Tamiflu. Temperatures for all groups remained close to baseline (day 0) throughout each day of the study, with no clear trend as observed in studies #2 and #3. (Figure 9A). Ferrets in all 4 groups demonstrated weight loss for the duration of the study, with the HB36.6 treated ferrets having the most weight loss (Figure 9B). Percentage of weight lost is similar for all ferrets until day 4 PI. Percentage weight loss in ferrets treated with Tamiflu plateaued on day 4 (at approximately -10%) and remains the same until necropsy. Untreated control ferrets increase in weight slightly from day 4 to day 5, then also plateau at about -12%. Ferrets treated with 20mg/kg on D1-5 and 20mg/kg at -2hr experience similar consistent percentage weight change throughout the study, ending at approximately -20% on day 6. The 20mg/kg HB36.6 at D1-5 treated group displayed the most severe clinical signs of disease throughout the duration of the study (Figure 9C). Statistics could not be determined due to a sample size of 1 (N=1) for the untreated control comparison.
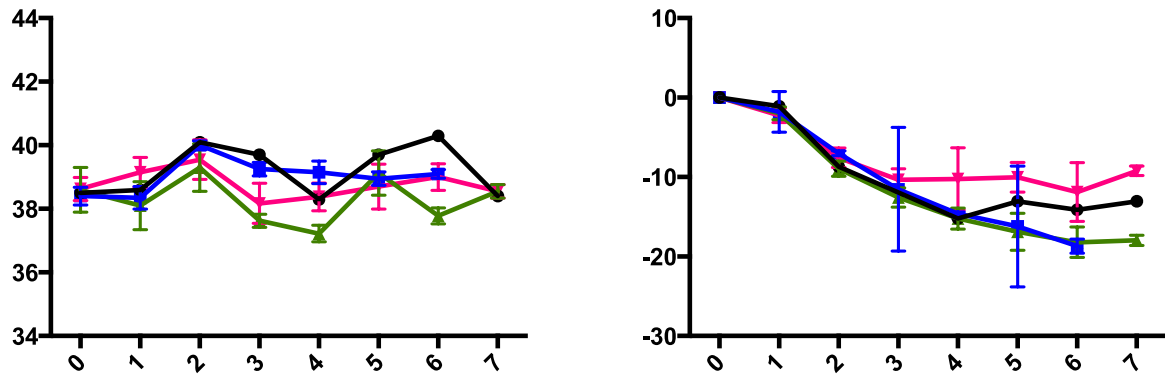
ey:

Figure 9. Clinical signs of disease in ferrets treated with a high dose of HB36.6 or Tamiflu, and untreated control ferrets after aerosol exposure to A/California/07/09.

Study #4 consisted of one untreated control ferret (black circles), two ferrets treated prophylactically with 20mg/kg HB36.6 two hours before infection with H1N1 (blue squares), four ferrets treated therapeutically with 20mg/kg on days 1-5 PI of the study (green triangle), and four ferrets treated therapeutically with Tamiflu (5mg/kg, twice daily

on days 1-5) (pink inverted triangles). Ferrets were challenged with 2.6×10^7 TCID$_{50}$ A/California/07/09. (A) Temperature (°C) of treated and untreated control ferrets over the course of infection. (B) Percentage of weight change of treated and untreated control ferrets over the course of infection. (C) Clinical scores of treated and untreated control ferrets over the course of infection. Graphs A and B are shown with mean and SD at each time point. Graph C is shown with mean at each time point. Statistics cannot be used to analyze these data due to a sample size of N=1 for the control.

4.1.4   Summary of Aim 1

Studies #2 and #3 showed that ferrets treated with HB36.6 displayed fewer clinical signs of disease compared to untreated control ferrets, and for study #2 these differences were significant. The data obtained in study #4 was complicated by several experimental problems, the most notable being that 2 of the 3 control animals did not become infected post influenza challenge. This was determined to be due to prior exposure to influenza from the facility/workers while in holding between studies #3 and #4 at the University of Pittsburgh. In addition, study #4 used a different stock of challenge virus resulting in a much higher delivered dose, and there were unexpected results with the Tamiflu treated controls. Finally, the high dose (20mg/kg) HB36.6-treated animals demonstrated some irritation to the drug alone, resulting in diarrhea and reduced appetite, complicating the results post infection. Altogether, it will be necessary to repeat study #4 to obtain reliable data that can be compared to studies #2 and #3.

4.2   AIM 2: TO DETERMINE WHETHER TREATMENT WITH HB36.6

REDUCES VIRAL TITERS IN NASAL WASH AND LUNG TISSUE SAMPLES

COMPARED TO UNTREATED CONTROLS FOLLOWING AEROSOLIZED

INFLUENZA INFECTION IN THE FERRET MODEL

The goal of Aim 2 was to further evaluate the virologic and immunological properties of the samples obtained in studies #2, #3 and #4. Measurement of viral loads by semi-quantitative reverse transcriptase RT-PCR is presumed to be reflective of infectious virus particles. Thus, a higher viral load is generally associated with more severe disease in the ferret model. To quantitate the virus in ferret samples post infection, nasal wash samples were collected daily and lung tissue samples were harvested at necropsy for evaluation.   RNA was extracted and semi-quantitative reverse transcriptase RT-PCR was performed to quantitate the viral copies. Data is displayed using dot plots with mean shown. Formal statistics were only completed for study #2, where groups consisted of 3 or 4 ferrets.

4.2.1   Study #2

Study #2 was designed to evaluate the ability of HB36.6 to reduce virus when given prior to infection at a low dose. RT-PCR on these study #2 samples was performed by Dr. Amy Hartman. Viral titers were highest in nasal wash samples for all groups on day 2 PI (Figure 10A). Untreated controls had the highest viral copy levels on days 1 and 2 PI; viral copies were reduced in the HB36.6 treated ferrets in a dose-dependent manner (10 mg/kg group had a mean viral load of 1.14 x 10^5 compared to the 2.5 mg/kg group having a mean viral load of 4.00 x 10^5 on day 2 PI) (Figure 10B). Similar results were observed in lung tissue, where the untreated

controls demonstrated the highest average viral titers (2.65×10^5), followed by the 10 mg/kg treated group (1.97×10^5), and the 2.5 mg/kg treated group having the lowest average amount of virus (3.84×10^3). Taken together these data suggest that HB36.6 is able to reduce the viral load when administered pre-infection at low doses.
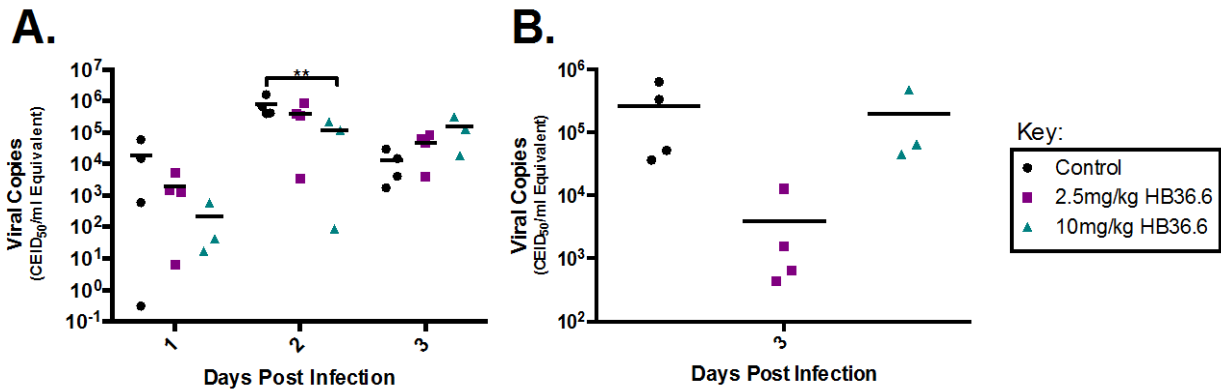
Figure 10. Quantification of viral loads in nasal wash and lung samples in ferrets treated with low doses of HB36.6 and untreated control ferrets after aerosol exposure to influenza virus A/California/07/09.
Study #2 consisted of four untreated control ferrets (black circles), four ferrets treated with 2.5mg/kg HB36.6 two hours before infection with pH1N1 (purple squares), and three ferrets treated with 10mg/kg HB36.6 two hours before infection (teal triangles). Ferrets were challenged with 2.6×10^7 TCID$_{50}$ A/California/07/09. (A) Nasal wash viral copies in ferrets groups after aerosolized infection with pH1N1, displayed as a dot plot to show individual ferret data. (B) Ferret viral copies detected in lung 4/6 on day 3 PI. Graphs are shown with mean at each time point. Statistical analysis on nasal wash data was performed using a two-way ANOVA for multiple comparisons.

4.2.2   Study #3

Study #3 evaluated high dose (20mg/kg) HB36.6 at two time points pre-infection to determine how early prior to infection the antiviral could be administered and still have an effect on blunting influenza infection and disease progression in ferrets. Evaluation of nasal wash samples from these ferrets demonstrated that virus peaked on day 3 PI (Figure 11A) with a mean

viral load ranging from 2.58 to 6.82 x 10^4 in untreated controls and HB36.6 treated groups, respectively. Differences in viral loads in lung tissue from treated animals compared to untreated controls were not as substantial as observed in study #2 and we actually observed less virus in the controls compared to the treated animals (Figure 11B). It should be noted that on day 3 PI most animals received fluids due to their level of dehydration. On day 5 PI we observed an increase in virus and a decrease in clinical scores, presumably due to the increase in level of hydration of the animals. However, most animals regressed and by day 6 their scores and viral loads returned close to their day 3 or 4 levels. There were two differences in study #3 compared to study #2 that are worth noting. First, study #3 was carried out to 6 days post infection while study #2 only lasted 3 days PI. This longer duration of study made comparison of the viral loads in lung tissue impossible, and given that all treated animals received antiviral treatment prior to infection, it is unlikely that the antiviral would be able to continue to inhibit virus for 6 days. Thus we are likely seeing an increase in virus replication after 2-3 days PI of virus that was not controlled by HB36.6 treatment. Second, the HB36.6 dose was higher in study #3 and some irritability was observed in the ferrets following administration prior to influenza infection. Regardless, study #3 did not demonstrate any major changes in viral loads in ferrets treated with HB36.6 administered within 24hr prior to influenza infection compared to untreated controls.
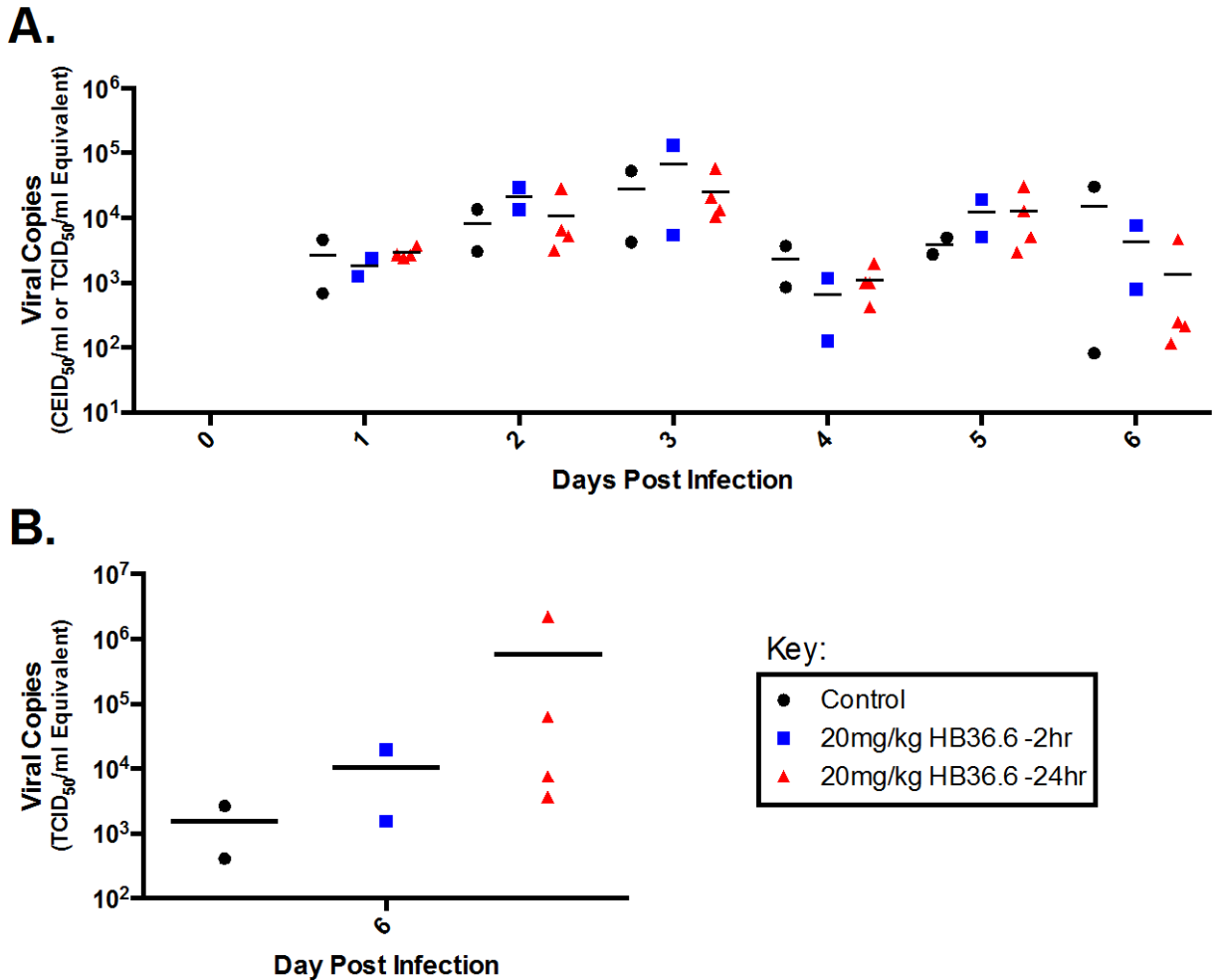
Figure 11. Quantification of viral loads in nasal wash and lung samples in ferrets treated with a high dose of HB36.6 and untreated control ferrets after aerosol exposure to influenza virus A/California/07/09.

Study #3 consisted of two untreated control ferrets (black circles), two ferrets treated with 20mg/kg HB36.6 two hours before infection with H1N1 (blue squares), and four ferrets treated with 20mg/kg HB36.6 24 hours before infection (red triangles). Ferrets were challenged with 2.6×10^7 $TCID_{50}$ A/California/07/09. Viral loads were measured by semi-quantitative reverse-transcriptase RT-PCR following aerosolized pH1N1 infection in (A) nasal wash, and (B) lung tissue at necropsy. All data is represented with the mean at each time point. Statistics could not be analyzed with these data due to a sample size of N=2 in some groups.

4.2.3 Study #4

Study #4 set out to further evaluate HB36.6 therapeutically and compare it to the standard of care, Tamiflu. For this study, ferrets were treated with a high dose (20mg/kg) of HB36.6 before or after aerosolized influenza infection and compared to untreated controls and Tamiflu treated ferrets. Viral loads were again measured in longitudinal nasal wash samples and lung tissue from necropsy. Due to experimental issues, 2 of 3 untreated control ferrets failed to become infected leaving the study with inadequate controls. Further, the high dose of HB36.6 resulted in more evident irritability in the ferrets with observed loss of appetite and diarrhea following each dose. As a result of these issues, viral loads in nasal washes were only slightly different among the treatment groups (Figure 12A). Interestingly, while differences were not as striking as earlier experiments, there was an overall trend toward decreasing viral loads in nasal wash samples early after infection (day 3) in HB36.6-treated ferrets. These data show that treatment prior to infection was just as effective as daily treatment on days 1-5 after infection, though these differences were not substantial. Further, the lung samples at necropsy did not mirror the nasal wash results (Figure 12B). It will be necessary to repeat study #4 to obtain reliable data.
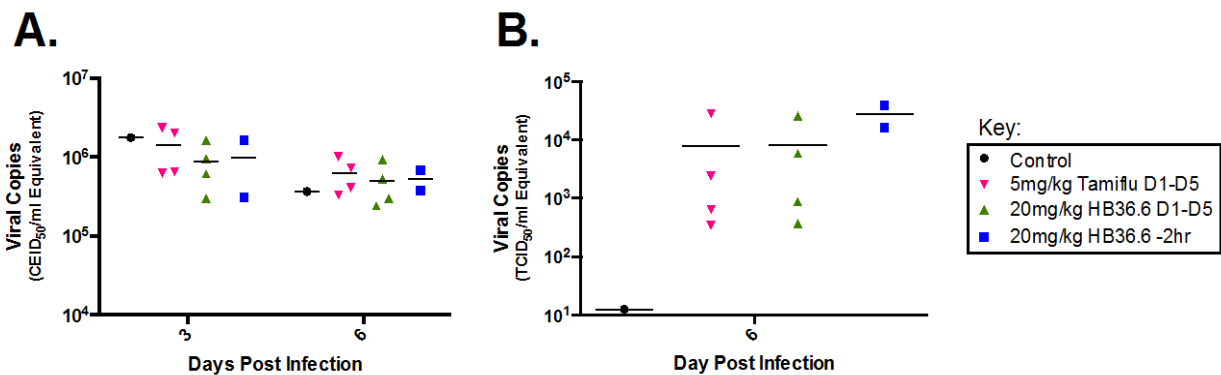
Figure 12. Quantification of viral loads in nasal wash and lung samples in ferrets treated with a high dose of HB36.6 or the recommended dose of Tamiflu, and untreated control ferrets after aerosol exposure to influenza virus A/California/07/09.

Study #4 consisted of one untreated control ferret (black circles), two ferrets treated prophylactically with 20mg/kg HB36.6 two hours before infection (blue squares), four ferrets treated therapeutically with 20mg/kg on days 1-5 PI (green triangles), and four ferrets treated therapeutically with Tamiflu (5mg/kg, twice daily on days 1-5) (pink inverted triangles). All Ferrets were challenged with 2.6 x 10^7 $TCID_{50}$ of aerosolized influenza A/California/07/09, and viral loads were measured in (A) nasal washes on day 3 and 6 PI, and (B) lung tissue at necropsy. Data is represented with mean at each time point. Statistics could not be analyzed in all groups due to low sample numbers [control (N=1) and 20mg/kg −2hr groups (N=2)].

4.2.4   Summary of Aim 2

When administered before infection, HB36.6 demonstrates some differences in viral loads in nasal wash and lung tissues early on in infection. These data are complicated from issues experienced with higher concentrations of the antiviral HB36.6, especially when the binder is administered daily, as the ferrets demonstrated symptoms uncommon with influenza infection, including severe diarrhea and loss of appetite. We believe that HB36.6 may only be

helping to block infection for up to day 3 PI, after which the virus is able to replicate more freely and differences between the treated and untreated control ferrets starts to narrow. These longer studies need repeated with higher sample sizes to see statistical differences.

4.3   CYTOKINE DETECTION IN BLOOD AND PLASMA SAMPLES

Cytokines induced by innate immune responses contribute to more severe clinical outcomes in disease. The Cole lab has previously used semi-quantitative RT-PCR to measure specific cytokines in tissue samples, standardized to a housekeeping gene. However, it is more desirable to be able to measure these responses in the periphery (nasal wash and blood) since these samples are easier to acquire, and are more realistic samples to study for use in humans. Thus, we are currently working to develop a peripheral assay to detect cytokines in ferret blood/plasma samples following experimental influenza infection. We have attempted to measure cytokines using an enzyme-linked immunosorbent assay (ELISA) and quantitative real-time RT-PCR. For the ELISA, we tried identifying cross-reactive reagents from other species, including human, mouse and nonhuman primate. Other species that have become of interest and demonstrated cross-reactivity include the dog and cat (we have not yet tried). We also identified a lab working to generate ferret-specific reagents and contacted them for their assistance. We obtained two protein/antibody combinations donated to the NIH BEI Resources Repository from this PI, and after multiple attempts, could not get these reagents to work by ELISA. We were most disappointed to learn that BEI uncovered the fact that the antibodies were not ferret, as promised. We have been able to perform the cytokine RT-PCR and may use this for future analyses of the samples.

4.3.1 ELISA

We obtained ferret protein-antibody pairs from Dr. David Kelvin in Canada who published on the generation and use of these reagents. We worked on the development of a new ELISA to detect cytokines in the periphery as a comparison to tissue RT-PCR. Using serum and blood (pre-bleed) samples obtained from study #2, we attempted to develop a ferret cytokine array using a protocol developed from information provided in [11] to measure IFN-γ, shown in Appendix A. However, we were unable to replicate the Kelvin lab's results.

Compared to the blank wells filled with an equivalent volume of blocking solution (1% BSA in PBS), there was very little antigen present in the samples in Table 1 using ELISA procedure #1 (Appendix A) because the optical density (OD) readings were similarly low. The pre-bleed sample had the highest amount of IFN-γ detected, with 0.120 at a 1:10 dilution, followed by the serum sample with 0.096 for the 1:10 dilution. The serum 1:40 and 1:80 dilutions must have been switched.

Table 1. IFN-γ Detection in Ferret Serum and Blood Samples Using ELISA Protocol #1

Sample	Task	Conc/Dil	OD (450nm)
M-AB 4A4B7	+ Control	0.05	0.069
M-AB 4A4B7	+ Control	0.025	0.066
M-AB 4A4B7	+ Control	0.0125	0.071
M-AB 4A4B7	+ Control	0.00625	0.075
M-AB 1H1H12	+ Control	0.05	0.067
M-AB 1H1H12	+ Control	0.025	0.066
M-AB 1H1H12	+ Control	0.0125	0.066
M-AB 1H1H12	+ Control	0.00625	0.068
Serum	Unknown	1:10	0.096
Serum	Unknown	1:20	0.086
Serum	Unknown	1:40	0.069
Serum	Unknown	1:80	0.076
Pre-bleed	Unknown	1:10	0.120
Pre-bleed	Unknown	1:20	0.092
Blank (Blocker)	- Control	0	0.067
Blank (Blocker)	- Control	0	0.064

In Table 2, the negative control and positive control had similar OD readings, so these results were not considered positive.

Table 2. IFN-γ Detection in Ferret Serum and Blood Samples Using ELISA Protocol #2

Name	Task	Conc/Dil	OD (450nm)	Count	Mean	Std Dev	CV (%)
IFN-γ Antigen	+ Control	0.1μg/well	0.071	2	0.069	0.004	5.16
IFN-γ Antigen	+ Control	0.1μg/well	0.066				
Serum	Unknown	1:10	0.081	2	0.081	0	0
Serum	Unknown	1:10	0.081				
Serum	Unknown	1:20	0.081	2	0.082	0.001	1.72
Serum	Unknown	1:20	0.083				
Blank	- Control	0	0.067	2	0.068	0.001	1.05
Blank	- Control	0	0.068				

4.3.2 Cytokine PCR

ELISA is the more common method seen in the literature for detection of cytokines in serum samples. However, real-time PCR methods also offer sensitive detection and accurate quantification of low levels of cytokine mRNAs, and a number of researchers continue to use this method [32, 33]. This method also includes the advantages of the ability to quantify multiple targets per well using different fluorochromes, which can reduce costs and time spent on experiments [33].

Table 3. Cytokine Detection PCR with Fuller Study #2 Samples

Primers	Sample	Detector	Task	Ct
GAPDH	Plasma #7, D2	SYBR	Unknown	12.55983
GAPDH	Plasma #7, Nec	SYBR	Unknown	Undetermined
GAPDH	Plasma #9, D2	SYBR	Unknown	Undetermined
GAPDH	Plasma #9, Nec	SYBR	Unknown	Undetermined
GAPDH	Lung #7	SYBR	+ Control	Undetermined
GAPDH	Lung #9	SYBR	+ Control	Undetermined
GAPDH	NTC	SYBR	- Control	Undetermined
GAPDH	RT-	SYBR	- Control	Undetermined
IL-10	Plasma #7, D2	SYBR	Unknown	Undetermined
IL-10	Plasma #7, Nec	SYBR	Unknown	Undetermined
IL-10	Plasma #9, D2	SYBR	Unknown	Undetermined
IL-10	Plasma #9, Nec	SYBR	Unknown	34.69907
IL-10	Lung #7	SYBR	+ Control	36.87696
IL-10	Lung #9	SYBR	+ Control	Undetermined
IL-10	NTC	SYBR	- Control	Undetermined
IL-10	RT-	SYBR	- Control	Undetermined
IFN-γ	Plasma #7, D2	SYBR	Unknown	26.64936
IFN-γ	Plasma #7, Nec	SYBR	Unknown	Undetermined
IFN-γ	Plasma #9, D2	SYBR	Unknown	Undetermined
IFN-γ	Plasma #9, Nec	SYBR	Unknown	Undetermined
IFN-γ	Lung #7	SYBR	+ Control	Undetermined
IFN-γ	Lung #9	SYBR	+ Control	Undetermined
IFN-γ	NTC	SYBR	- Control	4.280223
IFN-γ	RT-	SYBR	- Control	Undetermined

In a limited set of experiments to detect cytokine mRNA in our ferret samples, RNA was extracted using the RNeasy® Mini Kit with on-column DNase Digestion (Qiagen, cat. #774104 and #79254). First strand cDNA synthesis was performed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat. #K1642), and two-step quantitative real-time RT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, cat. # K0221). PCR results were inefficient at best, and the metabolic enzyme GAPDH, used as a housekeeping gene in these experiments, was not reliably detecting a signal in all tissues as expected. In addition, the only cytokines detected were IFN-γ in plasma #7, D2 and a very small

amount of IL-10 in Plasma #9, Nec and Lung #7 (Table 3). We attempted RNA extractions using the PureLink Viral RNA/DNA Kit (Invitrogen, cat. #12280-050) and QIAamp® Viral RNA Mini Kit (Qiagen, cat. #52904) with no additional success. The PCR needs to be optimized with known positive samples and then these tissues repeated. Other housekeeping genes in ferret tissues and whole blood samples will also be evaluated as recent literature has reported inconsistencies using GAPDH, especially in certain tissues or during disease states [34].

5.0   DISCUSSION

Evaluation of HB36.6 in the ferret model confirmed studies in mice, demonstrating that treatment with this novel antiviral in low to moderate doses (2.5-10 mg/kg) within 24hr prior to aerosolized infection resulted in reduction in peak viral load and decreased severity of disease. Attempts to increase the dosage resulted in irritation and possible toxicity in the ferrets, displayed by their diarrhea and loss of appetite following each dose. It will be important in future studies to determine if there is indeed toxicity from the antiviral through pathology or other testing, and to seek alternate formulations that will be less irritating without compromising the antiviral potency.

Studies #2 and #3 afforded important clinical information about the antiviral capabilities of HB36.6. When looking at the data between the two studies, the results demonstrated a similar trend of inhibiting viral loads when HB36.6 was administered with 24 hours before infection. However, there were also some differences between the studies that warrant further evaluation. First, the two studies were designed with different endpoints: study #2 ended on day 3 PI while study #3 did not end until day 6 PI.   This is an important distinction since in both studies the peak viral load appeared between 2-3 days PI, yet study #3 allowed the animals to progress without further antiviral treatment. This in turn provided time for the virus that was inhibited by HB36.6 to begin replicating and, for our analyses, to reflect increases in virus titers. Thus, future

studies should either be shorter to evaluate reduction in peak viral load, or combine prophylactic and therapeutic treatments for longer studies.

In study #2, viral titers did not mimic clinical scores, as viral copies were highest on day 2 PI, and were beginning to decrease in two groups (untreated controls and 2.5mg/kg treated) by day 3 PI, where clinical scores on day 3 were still increasing. This could indicate that clinical signs of disease need time to catch up with a change in viral loads and there is a slight delay in feeling sick. Similar titer amounts were found in the untreated control ferret nasal wash and lung samples. Slightly higher amounts of viral copies were found in the untreated control group lungs (~2.65 x 10^5) compared to their nasal wash (~1.26 x 10^4) titers. There was an approximate one log_{10} difference between viral copies in the lung (~3.84 x 10^3) and nasal wash (~4.86 x 10^4) samples of the 2.5mg/kg treated ferrets on day 3 PI. Ferrets in study #2 received an average inhaled dose of 879 $TCID_{50}$; this was the lowest average inhaled dose amongst the three studies.

Contrary to study #2, the untreated control ferrets in study #3 had the greatest weight loss over the course of infection, and they were also the group with highest average starting weights. One ferret nearly reached the requirements for euthanasia by reaching 29.6% weight loss on day 3 PI. This ferret was given 10cc subcutaneous fluids, was watched carefully for a few hours after the alarming recorded drop in weight, and had improved when reweighed some time later. Similarly to study #2, in study #3 clinical scores were higher for the untreated control group, but this did not happen until later into the course of infection (days 4-6) where with lower doses of HB36.6, ferrets demonstrated differences in clinical scores by day 2 PI. Several of the ferrets received 10cc subcutaneous fluids at day 3 PI, including both control ferrets [10mg/kg at −2hr, N=1; 20mg/kg at −24hr, N=2]. These fluids likely influenced the severity of disease exhibited on day 4 PI and help to explain the dip in nasal wash viral loads seen the following day.

Reduced viral titers were detected in nasal wash samples on day 6, but not in lungs. Ferrets in this study received an average inhaled dose of 1,711 TCID$_{50}$. It is likely that a combination of a much higher inhaled dose and prolonged study time contributed to the results, potentially masking early antiviral benefits (nasal washes only were available for evaluation; no lungs were harvested until necropsy).

In study #4, ferrets treated with the experimental binder HB36.6 gradually lost the most weight by the end of the study, as where the Tamiflu and untreated control groups started to plateau in weight at days 4 and 5 PI. Clinical scores for this study revealed ferrets that received a high dose of HB36.6 daily displayed higher clinical signs of disease, and these scores were consistent for days 2-6 PI of the study. Two 20mg/kg daily (D1-5) HB36.6-treated ferrets were not sacrificed until day 7 PI, and were beginning to show signs of recovery on this day. We suspect that since the ferrets had not been given binder since day 5, by day 7 they were beginning to recover from its toxic affects. However, our study results are unreliable because we only had N=1 for an untreated control comparison, and Tamiflu did not seem to help lessen clinical signs of disease, contrary to the literature. In an article published last year, funded in part by the World Health Organization (WHO), researchers concluded that treating influenza-infected ferrets with oseltamivir (Tamiflu) is associated with improved clinical outcomes, including the ferrets remain more active, lose less weight, and experience a smaller rise in body temperature throughout the course of infection, despite no significant reduction in viral loads [35]. Viral loads on day 6 PI were higher in nasal wash ($\sim$3.68-6.23 x 10^5) than lung samples ($\sim$1.25 x 10^1 for controls and $\sim$7.92 x 10^3 – 2.79 x 10^4 for treated animals). Ferrets received an average inhaled dose of 4,818 TCID$_{50}$; this dose was logs higher than those in the previous studies.

These three studies with HB36.6 are not directly comparable because different stocks of virus were used for each study, the studies took place over varying time points, and the controls did not act the same between experiments. One reason for differences in clinical scores within studies could be due to the subjective nature of manually completing clinical scoring sheets. The scoring sheets were primarily completed by Jeneveve Lundy, a DLAR veterinary technician, for studies #2 and #4, while study #3 scores were primarily reported by the Cole lab. WHO researchers have reported that using a video tracking method to evaluate the effects of influenza infection and treatment on ferret activity is more sensitive at detecting activity changes, and less subjective, than manual scoring [35]. The doses of virus used to infect ferrets were very high to ensure all ferrets became infected. Fuller ferret study #1, which is not discussed as part of this project, was a titration study of the influenza virus using 2 ferrets infected with a high dose of influenza (undiluted stock, inhaled dose of 12,620 $TCID_{50}$), 2 ferrets infected with a low dose of influenza (stock 1:10 dilution, inhaled dose of only 201 $TCID_{50}$) and 2 control ferrets (sham aerosol, PBS only). Given the large difference in doses and the lack of differences in clinical results between the two groups of animals, we chose to move forward with undiluted challenges so as not to drop below the median lethal dose (LD_{50}). The average required dose to infect humans with influenza by aerosol challenge is 0.6-3 $TCID_{50}$. Fifty-percent ferret infectious dose (FID_{50}) is estimated to be as low as 10-30 $TCID_{50}$ for pre-pandemic H1N1 and 3 plaque-forming units (pfu) for a pandemic H1N1. In hindsight, we could have likely challenged with a 1:10 stock of virus. It is possible that by challenging with the undiluted stock, we used an overwhelming amount of virus and potentially limited our ability to observe the effect of antivirals. Research has shown in a mouse model that animals challenged with a low dose ($10^{3.5}$

EID$_{50}$) of influenza A virus can benefit fully from an antiviral that does not protect mice

challenged with a higher dose ($10^{6.8}$ EID$_{50}$), and Marriot *et al.* found similar data in ferrets [36].

From study #2, we concluded that low dose prophylactic treatment with HB36.6 is

effective at reducing clinical signs of disease in ferrets infected with influenza, but there is little

to no effect on viral loads. This is similar but not as pronounced in study #3. We speculate that,

in addition to the high dose challenge, this may be related in part to immune responses such as

cytokine induction that differed in ferrets treated with HB36.6 compared to untreated controls.

In conclusion, using prophylactic doses of HB36.6 seems to reduce clinical signs of disease in

ferrets infected with influenza, but has less effect on viral loads. Further studies are needed to

determine the timing and dosage for optimal treatment with HB36.6 as a successful therapeutic

against aerosolized influenza A infection.

5.1   PROPOSED MODEL

If this ferret model accurately mimics H1N1 influenza A infection and treatment in

humans, then this new antiviral could work similarly to oseltamivir (Tamiflu). Professor Chris

Del Mar, MD, a member of Cochrane who helped analyze Tamiflu data, expressed his concerns

with the antiviral saying that it could help people feel better, but does not reduce viral shedding

[37]. This increases the chances of a public health disaster because people will feel better and

think that they have cleared the virus, so will be more likely to be out in the community and still

be able to pass the virus onto others. HB36.6-treated ferrets in studies #2 and #3 displayed fewer

clinical symptoms, yet did not show much difference in viral copy numbers at necropsy on days

3 or 6 PI. HB36.6 is administered intranasally and has been hypothesized to coat the nasal tract

as one mechanism to block infection. It is possible that the toxicity effect on daily HB36.6-treated animals in study #4 actually helped to increase viral copy numbers detected in the nasal washes instead of blunting the viral infection as intended.

Cytokine analysis to look at immunologic response throughout the course of infection would be helpful in further shaping our proposed model. Additional studies with larger animal numbers also need to be completed to increase sample size and provide statistical significance. However, based on this limited data, we propose the following model (Figure 13): administration of a prophylactic dose of HB36.6 helps coat the nasal tract beginning in the nares and following down the trachea into the lungs; as virus is inhaled it is bound by the antiviral and the overall initial inoculum is lessened, decreasing the initial insult to the host; the early clinical signs of disease following aerosol infection are thus lessened when compared to untreated animals. However, in the absence of additional treatment, virus that does get inside the animal begins to replicate and recruit immune cells, causing activation and an increase in virus replication. Therefore, after the first few days it is no longer possible to see differences in either the clinical signs or viral loads in treated or untreated animals. Repeating study #4 with low-dose therapeutic treatment of HB36.6 may provide better protection, helping to keep the viral burden and clinical signs of disease reduced. Regardless, it is likely that in the current studies, differences in clinical signs of disease are associated with differences in immunologic responses exhibited throughout the course of infection.
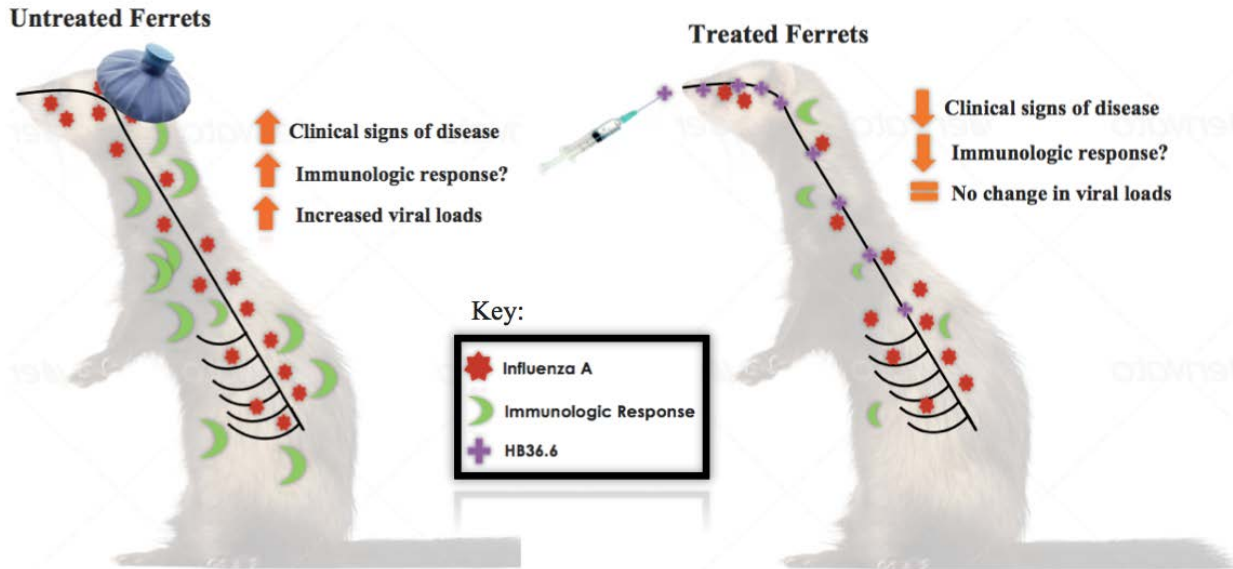
Figure 13. Proposed model for treatment with low dose HB36.6 after aerosol exposure to influenza virus A/California/07/09.

We assume that this ferret model mimics what will happen in human trials. Untreated and HB36.6-treated (purple plus-sign) ferrets showed similar viral titers (red 7-point star) in nasal wash and lung samples. Treated ferrets display fewer signs of disease, which may be associated with reduced immunological responses (green half-moon).

5.2   PUBLIC HEALTH SIGNIFICANCE

Influenza virus causes an estimated 250,000-500,000 deaths annually and remains a significant public health issue. When a host is infected with multiple strains of influenza A, gene reassortment can occur leading to new subtypes of the virus that cause increased morbidity and mortality in the human population [5]. Influenza also has a high evolutionary capacity, which quickly leads to drug-resistance against several FDA approved antiviral drugs, as seen with oseltamivir (Tamiflu) [3]. The best protection against influenza infection is receiving the annual vaccine. However, due to the current selection for strains that go into the annual vaccine, and the

lead time necessary to produce the vaccine annually, the vaccine is not always well-matched or effective against the following influenza season. There are also certain subsets of the population that are either unable to receive the vaccine, or are more susceptible to disease, including the very young and elderly, immune-compromised individuals, and pregnant women. Therefore, it is pertinent that more effective antivirals, that have not yet induced antiviral resistance, come to market.

Small molecular proteins have great potential for clinical use, as they can help shorten disease duration and reduce the risk of influenza-associated complications, as well as death, if taken in a timely manner. Influenza A viruses consist of eight segmented RNA genes that encode 12 known proteins: HA, NA, PA, PB1, PB1-F2, PB2, NP, N40, M1 matrix proteins, M2 ion channel proteins, NS1 and NS2, all of which have potential as drug targets. Combination therapies using antivirals with different mechanisms of action can also be used to achieve greater efficacy. For example, a co-treatment with amantadine + oseltamivir, or favirpiravir + oseltamivir has demonstrated better efficacy against H5N1-challenged mice. Treatment with three antiviral drugs, amantadine + ribavirin + oseltamivir, helped reduce mortality in Korean patients hospitalized with severe A/H1N1 2009 (pH1N1) [17]. This project explores the use of a new HA-binding antiviral protein for group 1 influenza A viruses that can be used on its own or as a combination treatment with other anti-influenza drugs. The advantages of using small molecular proteins include that they are typically more stable, less immunogenic, and less costly than larger molecular drugs [38]. We hope that these advantages will allow great success in using small proteins as anti-influenza treatments and help reduce the morbidity and mortality associated with this disease.

5.3   FUTURE DIRECTIONS

Given the high clinical scores in the therapeutically treated ferrets with a high dose of HB36.6 compared to untreated controls, a study should be done in the ferret model without viral infection to assess toxicity of the antiviral protein. A second, even broader-binding antiviral peptide is currently under development to include protection from group 2 subtypes of influenza A by combining a second small protein to work along with HB36.6. This would allow the antiviral to protect from up to 16 different subtypes of influenza as well as drifted and shifted genetic variants of the virus. Additional computationally-designed proteins are being evaluated for their ability to inhibit influenza B viruses.

The Cole lab will further evaluate ferret tissues for cytokine responses following experimental influenza infection, comparing antiviral treated and untreated samples.   Several new reagents are being evaluated currently for these assays, so that assays will be able to utilize blood and serum samples as well as RT-PCR. The success of this project will allow for better detection and understanding of the immunologic responses and specifically cytokine responses elicited by influenza infection, and give rise to better reagents for the ferret model. For example, researchers have reported that ferret inflammatory cytokines share a similar specific structure to humans, including IFN-γ, TNF-α, IL-8, IL-6, and IL-1β, [32]. However, not many reagents have been successfully shown to cross react with the ferret. The Cole lab has recently partnered with the Center of Excellence for Influenza Research at St. Jude Children's Research Hospital to obtain newly developed ferret reagents that will be used in these studies.

The goal is to continue to monitor cytokine responses by RT-PCR and adapt new assays to measure proteins in the periphery (blood and nasal wash samples) so that the translation of data between the ferret model and the human is more relevant. These studies will provide

information on the relevance of immune responses elicited in the ferret during influenza infection, so that this model can further be used to predict and evaluate how to overcome the cytokine storm and reduce fatal outcomes in humans.

A.1   VIRUS PROPAGATION

A.1.1 MDCK and Hela Cell Lines
- **Materials/Reagents:**
  1. Madin-Darby Canine Kidney (MDCK) cells (ATCC CCL-34)
  2. HeLa cells (ATCC)
  3. Maintenance Media
       a. 500ml DMEM (1X)
       b. 5ml   Penicillin/Streptomycin (10,000 U/ml / 10,000 ug/ml)
       c. 5ml   HEPES Buffer (1M stock)
       d. 5ml   L-gluamine, 200 mM (100X), if needed
       e. 25ml   Heat Inactivated FBS
       f. 5ml   Sodium pyruvate, if needed (may already be in DMEM)
  4. Trypsin/EDTA, 0.25%
  5. Phosphate-buffered saline (PBS) (pH 7.2)
  6. Water bath
  7. T75, T75 and T150 flasks with vented cap
  8. 37°C, 5% CO_2 incubator
  9. 15ml conical tube
  10. Trypan Blue solution, 0.4% (Sigma, cat. #T8154)
  11. Hemocytometer
  12. 1.5ml cryovials
  13. Freezing media (90% FBS/10% DMSO)
       a. Fetal Bovine Serum (FBS) (Atlanta Biological, Cat. #S11150)
       b. Dimethyl Sulfoxide (DMSO) (Sigma, Cat. #D2650)
  14. Freezing Container, 1°C/hr (NALGENE™ Cryo, cat. #5100-0001)
  15. Alcohol
  16. -80°C freezer
  17. Liquid nitrogen storage
- **Protocol:**
  1. Propagation
       a. Thaw cells from LN2 in 37°C water bath
       b. Resuspend in 10ml maintenance media
       c. Centrifuge for 5 minutes at 400g in a 15ml conical tube

     d. Dump supernatant in bleach

     e. Resuspend in 8ml maintenance media

     f. Pipette cell suspension into a T25 flask with a vented cap

     g. Incubate for 24-36 hours in 37°C, 5% CO_2 incubator

2. Cell Splitting/Maintenance

     a. Cells should be split 1:10 every 3-4 days

     b. Warm maintenance media and trypsin to 37°C using water bath

     c. Wash cells twice with 10ml room temperature PBS

     d. Add 2ml trypsin (for a T75 flask; 4ml for a T150flask) and tilt flask to coat bottom of flask

     e. Place flask in incubator until cells round up and dislodge (5-10mins) when the flask is tapped from the side

     f. Resuspend cells in a 5:1 volume of maintenance media: trypsin washing residual cells from the flask surface

     g. Move 1:10 volume to a new flask

     h. Incubate flask in 37°C/5% CO2 incubator for 3-4 days

          i. Note: MDCK cells should not be carried for more than 20 passages (P20') for productive influenza replication

3. Freezing Back Cells

     a. Grow cells to 80-90% confluency (log phase growth) using steps 2b-f above

     b. Count viable cells using trypan blue and a hemocytometer

     c. Resuspend cells at 10^7cells/ml in freezing media

     d. Aliquot 1ml suspensions into cryovials

     e. Place inside a freezing vessel and store -80°C freezer for up to 72hours

     f. After 48-72hours transfer cells to liquid nitrogen (LN2) for long-term storage

A.1.2 Influenza H1N1 Virus Egg Infection

- **Materials/Reagents:**
  1. Embryonated Chicken Eggs, 9-12 days old
  2. Flashlight
  3. Needle 23 gauge, 1 inch
  4. Needle 18 gauge, 1 inch
  5. Disposable syringe
  6. 50ml centrifuge tube with 70% alcohol
  7. Alcohol wipes
  8. Sterile forceps
  9. Influenza virus
     - a. A/California/07/09 (H1N1) at 2.6 x 10^8 $CEID_{50}$/ml (BEI Resources cat. #NR13663)
  10. Sharps container
  11. Tape
  12. Egg incubator
- **Protocol:**

1. Candle the eggs everyday starting at D8. Mark with a pencil the air sac and main veins.
   a. Good egg: Observe embryo movement and see intact blood vessels around the egg.
   b. Bad egg: Egg has too many white spots, too few veins, or is all black.

| Good egg | Unfertile egg | Dead egg | Rotten egg |
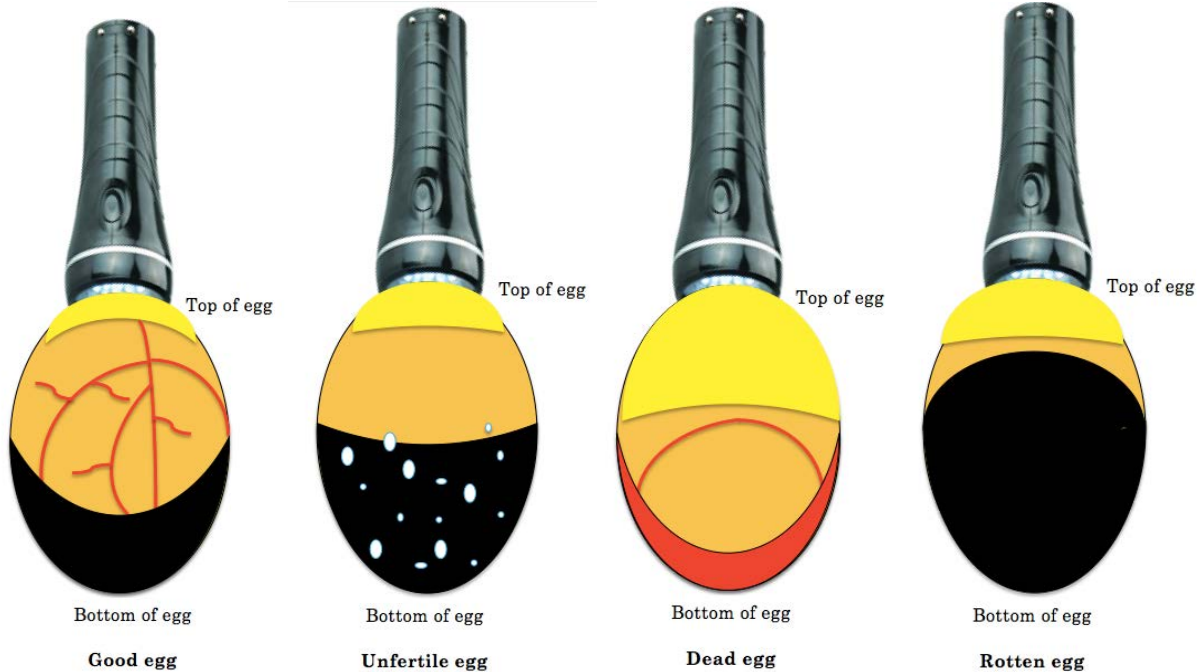
2. Inoculation of Influenza virus in embryonated eggs (usually use D11 eggs because has given best quality of fluid in the past: high volume of clear liquid).
   a. Prepare the virus inoculum: 10^{-3} – 10^{-10}, 10 eggs per dilution.
   b. Wipe top of eggs with alcohol wipes, let dry, and punch a small hole in the eggshell over the air sac using an 18 gauge, 1 inch needle. Make sure to punch hole close to fluid, so the needle can reach the fluid, and away from any major veins.
   c. Aspirate virus inoculum into 1ml syringe with 23 gauge, 1 inch.
   d. Insert the needle into the hole of the egg, pierce the allantoic membrane (about 0.5cm deep from egg shell) and inoculate 0.2ml per egg into the allantoic cavity.
      i. Change the syringe and needle when finished inoculating eggs in each tray or if the needle touches eggshell.
   e. Discard the syringe into a sharps container and seal the punched holes of the eggs with tape.
   f. Incubate the inoculated eggs at the appropriate conditions for virus strain.
      i. H1N1: 37°C, >80%RH humidity for 48 hours
   g. After 2 days of incubation remove the eggs from the egg incubator and candle to ensure eggs are still good.
   h. Chill the eggs by placing them in a refrigerator (4°C) overnight (at least 12 hours) or chill at -20°C for 2-3 hours to sacrifice the embryo and harden the blood vessels for good fluid harvest.

3. Harvesting the infected allantoic fluid
    a. Dip the forceps in 70% alcohol to sterilize and peel off the top of the eggshell (open up the air sac).
    b. Insert the 23 gauge, 1 inch needle downward into the allantoic sac.
    c. Draw out the clear allantoic fluid and avoid disturbing the blood vessels and yolk sac and place in proper container depending on dilution.
4. HA/Infectivity Test
    a. Perform an infectivity test to ensure titer. Usually get a titer >9 log EID50/0.2ml in the 10^{-6} – 10^{-9} dilutions, depending on virus strain and incubation conditions.

A.1.3 Hemagglutination (HA) Assay for Titering Influenza Virus
- **Materials/Reagents:**
  1. PBS (pH 7.2)
  2. V-bottom 96-well plate
  3. 5% Turkey red blood cells (TRBC) (Lampire Biological Labs, Pipersville, PA), fresh TRBC, less than 2 weeks old
      a. To make 0.5% TRBC:
          i. Spin 1ml TRBC at 650rpm for 5mins in 15ml tube
          ii. Dump supernatant and resuspend in 10mls of PBS
              1. Note: This is enough for two 96-well plates
  4. Multichannel pipette
  5. Influenza virus stocks
  6. Known positive virus for positive control
  7. Trough or reservoir
- **Protocol:**
  1. Pipette 50μl room temperature PBS per well in wells B through H
  2. Add 100μl virus to top wells (row A) in triplicate
      a. Needed Controls:
          i. Known positive virus control
          ii. Negative control = PBS in place of virus
  3. Add 50μl of row A to row B
  4. Add 50μl of row B to row C…continue as a 2 fold dilution (serial dilution, 1:2) down the plate, changing pipette tips in between each row
  5. When get to row H, remove 50μl and discard so total volume is the same (50μl)
  6. Add 50μl 0.5% TRBC to each well
  7. Agitate plate for 20 sec to mix samples
  8. Incubate for 1 hour at room temperature
  9. Observe agglutination reaction
      a. Button = no agglutination (negative reaction)
      b. No button = agglutination (positive reaction)
  10. Record endpoint HA titer, which is the last dilution to show agglutinations (last dilution with a positive reading)

A.1.4 TCID$_{50}$ Assay
- **Material/Reagents:**

1. Virus dilution media
    a. 500ml DMEM
    b. 5ml   Pen/Strep
    c. 12.5ml Bovine Serum Albumin (BSA) Fraction V, 7.5% in PBS
        i. 7.5g BSA in 100ml PBS
    d. 5ml   HEPES buffer (1M stock)
2. Viral growth media
    a. 100ml Viral Dilution Media
    b. 0.1ml 1:1000 2mg/ml TPCK-trypsin (2μg/ml) stock added at time of use
        i. Stock: dissolve 20mg TPCK trypsin (type XIII from bovine Pancreas) in 10ml dH_2O, sterile filtered through 0.2μm membrane, store at -20°C
3. PBS (pH 7.2)
4. Trypsin-EDTA, 0.25%
5. MDCK maintenance media
6. Trypan blue
7. Hemacytometer
8. 96-well flat-bottom plate or 96-well v-bottom plate
9. Microscope
10. 37°C, 5% CO_2 incubator

- **Protocol:**
  1. Prepare MDCK cells from a confluent T-75cm$_2$ flask
      a. Remove culture media from confluent T-75 flask
      b. Wash cells twice with 10ml PBS
      c. Add 2ml trypsin-EDTA to monolayer in flask
      d. Place flask in incubator until cells round up and dislodge (5-10mins) when the flask is tapped from the side
      e. Add 10mls maintenance media using multichannel pipette and swirl flask to get all cells off the sides
      f. Enumerate cell concentration using standard trypan blue and a hemocytometer (add 20μl cells + 20μl dye, and load 10μl of mixture onto hemocytometer)
      g. Dilute the cells to 3.0 x 10^5 cells/ml in maintenance media
      h. Dispense 100μl (3.0 x 10^4 cells/ml into each well of a 96-well plate (analyze up to 2 samples per plate)
      i. Incubate plate in a 37°C, 5% CO_2 incubator for about 24-30 hours, until a confluent monolayer is formed in the bottom of the wells
      j. Once monolayer is present, aspirate media from MDCK cells
      k. Wash cells twice with 200μl of PBS using multichannel pipette and remove using aspiration
      l. Add 150μl viral growth media to each well containing cells and set plate aside
  2. Set up virus titrations
      a. Obtain new, empty 96-well plate to make virus dilutions
      b. Add 180μl of viral growth media to rows B-H of 96-well plate

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      c. Add 200μl virus sample to row A (sample 1 = columns 1-5; sample 2 = columns 6-10; column 11 = known positive control; column 12 = negative control, which is 200μl of assay buffer only)

      d. Transfer 20μl of row A to row B and discard tips

      e. Mix 20 times using swirling tip action, transfer 20μl sample from row B to row C, and discard tips

      f. Continue the serial dilution down the plate, changing tips in between each row

3. Add virus dilutions to MDCK cells

      a. Using a multichannel pipette, transfer 100μl of each virus dilution to the MDCK cells in 96-well plate starting at the lowest concentration in row H, and working upwards using the same tips to mix

      b. Incubate plate at 37°C, 5% CO_2 incubator overnight

      c. Observe and record cytopathic effect (CPE) daily using the following key:

          i. CPE 0 = no cytopathic effects observes, same as controls

          ii. CPE 1 = monolayer intact, rounded up cells on top monolayer with few dead cells

          iii. CPE 2 = many dead cells, but monolayer intact

          iv. CPE 3 = most cells destroyed, but some remnants of monolayer observed

          v. CPE 4 = monolayer completely destroyed

      d. Calculate the $TCID_{50}$ using Reed-Muench method [39]

A.1.5 Hemagglutination Inhibition (HI or HAI) Assay for Detection of Influenza Specific Antibodies

- Materials/Reagents:
  1. See HA assay above for materials/reagents
     - a. See HA assay above for preparation of 0.5% TRBC
  2. Prepare 1% TRBC
     - a. Spin 1ml TRBC at 650rpm for 5 min in 15ml conical tube
     - b. Dump supernatant and resuspend in 10ml PBS for 1% working concentration
  3. Water bath
  4. Ferret sera samples
  5. Sterile microcentrifuge tubes
  6. Microcentrifuge
- Protocol:
  1. Prepare ferret sera
     - a. Heat inactivate ferret sera samples in 56°C water bath for 30min
     - b. Transfer 50μl heat inactivated sera to new tube
     - c. Add 200μl 0.5% TRBC suspension to each sera sample
     - d. Incubate the sera: TRBC mixture at room temperature for 30mins, vortex tubes every 10mins to mix
     - e. Pellet the TRBC in the sera tubes by microcentrifuging at 14,000rpm for 5sec

f. Transfer the sera into a sterile 96-well plate, being careful not to disturb the TRBC pellet
2. Perform HI assay
    a. See plate set-up below
    b. Pipette 50µl room temperature PBS per well in wells B-D and F-H of 96-well plate using a multichannel pipette and reservoir basin
    c. Add 100µl of each serum sample in duplicate to top wells (row A and E)
        i. Needed Controls:
            1. Known positive antigen control (diluted in duplicate in rows A-D)
            2. Negative control = PBS in place of virus (diluted in duplicate in rows E-H)
    d. Add 50µl of row A to row B
    e. Add 50µl of row B to row C, and row C to row D (2-fold dilution, 1:2 serial dilution), changing pipette tips in between each row
    f. Repeat serial dilution for rows E-H
    g. When get to rows D and H, remove 50µl and discard so total volume is the same (50µl) in all wells
    h. Dilute H1N1 virus stock to 4 hemagglutination units (HAU) (1:4 dilution)
        i. For 2 plates, dilute 3ml virus in 9ml PBS
    i. Add 50µl diluted virus stock to each well starting with row D and working upward to row A, mixing by pipetting, and repeat for rows H-E
    j. Agitate plate for 20sec to mix samples
    k. Incubate for 30min at room temperature
    l. Add 50µl of the 1% TRBC suspension to all wells starting with row H and working upward to row A, using new pipettes for each row
    m. Incubate plate for another 30-45 mins at room temperature
    n. Observe agglutination reaction
        i. Button = no agglutination (anti-H1N1 antibodies are present, Ab+; previously exposed to influenza)
        ii. No button = agglutination (no antibodies in serum, Ab-; no pre-exposed to influenza)

Table 4. Plate Set-up for HI Assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	+1:10	+1:10
B											+1:20	+1:20
C											+1:40	+1:40
D											+1:80	+1:80
E	S6	S6	S7	S7	S8	S8	S9	S9	S10	S10	-1:10	-1:10
F											-1:20	-1:20
G											-1:40	-1:40
H											-1:80	-1:80

Note: S# = sample #

A.2   FERRET CLINICAL SCORING OBSERVATIONS

Parameter		Degree of Parameter	Date:							
Initials										
Time of Observation										
Weight (Kg)										
Temperature (C)										
			Score							
Appearance	Posture	Normal	0							
		Hunched or abnormal posture	5							
		Prostrate	10							
	Activity	Alert and playful	0							
		Alert but playful only when stimulated	2							
		Alert but not playful when stimulated	5							
		Neither alert nor playful when stimulated	10							
	Skin/Eyes	Normal	0							
		Piloerection	2							
		Ocular Discharge	5							
Movement	Activity	Normal	0							
		Lethargy	5							
		Moribund	10							
	Neurological	Normal	0							
		Tremors	10							
		Hind limb weakness /paralysis	10							
Pulmonary Function	Respiration	Normal	0							
		Sneezing	2							
		Shallow or Rapid	2							
		Open mouth breathing	5							
		Labored/coughing	10							
Consumption/ Elimination	Food/water	Normal	0							
		Dehydrated	5							
		Decreased	2							
		Inappetant	5							
		Anorexic	10							
	Feces/Urine	Normal	0							
		No stool	2							
		Mucoid/Soft stool	2							
		Diarrhea	5							
		Bloody feces	10							
	Vomiting	No	0							
		Yes	10							
Total Score										
Comments (clarify any abnormality not defined by parameter description)										Date/ Initial

Note: A score of 0 indicates normal behavior or no display of signs of disease, where a higher clinical score is indicative of greater clinical signs of disease.

A.3   VIRAL TITER QUANTIFICATION

A.3.1 RNA Extraction
1. RNA Isolation from Liquid Samples (Blood, Serum, Supernatant, Nasal Wash)
   a. **Materials/Reagents:**
      i. TRI Reagent® Solution (Ambion, cat. #AM9738)
      ii. PureLink Viral RNA/DNA Kit (Invitrogen, cat. #12280-050)
      iii. 96-100% ethanol
      iv. 70% ethanol
      v. RNase/DNase-free microcentrifuge tubes
      vi. Class II Biological Safety Cabinet (BSC)
      vii. Chloroform (EMD™, cat. #CX1055-14)
      viii. RNase Away (Life Technologies, Thermo Scientific, cat. #10328011)
   b. Preparation of reagents:
      i. Add 60ml 96-100% ethanol to wash buffer in kit
   c. **Protocol:**
      i. Inactivation of samples:
         1. Add 100µl sample to 900µl TriReagent
         2. Mix by inverting or pipetting (DO NOT VORTEX)
         3. Let sit at room temperature for 5 mins
         4. Samples can now be brought out of the RBL by transferring to a new sterile Eppendorf tube, spraying the outside of the tubes with vesphene, double bagging and removing from the RBL
         5. Store the sample at -80°C in BSL2 or proceed immediately to extraction
      ii. Extraction and purification of RNA
         1. If samples were frozen, let thaw at room temperature
         2. Inside BSC, add 200µl chloroform to all samples and invert vigorously for 20sec (DO NOT VORTEX)
         3. Let sit at room temperature for 3mins
         4. Centrifuge at 12,000 x g for 15mins at 4°C
         5. Transfer 500µl of the aqueous phase (top clear layer) to a new tube
         6. The rest of the procedure can be performed on a benchtop, clean the benchtop and all supplies with RNase Away before proceeding
         7. Add 500µl 70% ethanol to all samples, vortex, and let sit at room temperature for 5mins
         8. Transfer the lysate to the viral spin column in a collection tube (supplied) in two 500µl increments (viral spin column can hold 700µl volume max)
         9. Centrifuge at 6800 x g for 1min
         10. Discard the collection tube and place spin column in a new collection tube (supplied)
         11. Add 500µl wash buffer with ethanol (prepared above, supplied by kit) to all viral spin columns

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12. Centrifuge at 6800 x g for 1min and discard the flow through
13. Repeat step 11-12
14. Discard collection tube and place viral spin column in new collection tube
15. Centrifuge at max speed for 1 min to dry the viral spin column
16. Place the viral spin column inside a clean Eppendorf tube (supplied by kit)
17. Elute RNA with 40µl sterile RNase-free water (supplied) directly to the viral spin column cartridge
18. Incubate samples at room temperature for 1min
19. Centrifuge at max speed for 1min
20. Discard the viral spin column and store the purified viral RNA at −80°C

2. RNA Isolation from Tissue Samples
   a. **Materials/Reagents:**
      i. TriReagent
      ii. chloroform
      iii. RNeasy Mini Kit (Qiagen, cat. #74104)
      iv. RNase-free DNase Set (cat. #79254) for on-column DNase digestion
      v. DMEM with 2% FBS
      vi. Balance (for measuring tissue weight)
      vii. Tissue Homogenizer (Omni TH) with Omni Tip™ Plastic Probes (Kennesaw, GA)
      viii. 5ml snap-cap tubes
      ix. Sterile forceps and scissors
      x. Vesphene
      xi. 70% ethanol
      xii. Bucket with ice
      xiii. Eppendorf tubes
      xiv. Vortex
      xv. -80°C Freezer
      xvi. Sorvall Legend Micro 17R/21R Centrifuge (Thermo Scientific)
      xvii. Class II Biological Safety Cabinet (BSC)
   b. Protocol:
      i. Homogenize Tissue
         1. Thaw tissue samples on ice and keep on ice throughout process
         2. Pipette 2x the volume (of tissue) of DMEM with 2% FBS (800µl media for 0.4g tissue)
            a. Note: For tissues weighing ≤200mg (0.2g), use 400µl media
         3. Under BSC, remove tissues from original vials using sterile forceps, weigh tissues to ≈400mg (0.4g) using balance and sterile scissors
         4. Transfer weighed tissue to 5ml snap-cap tube containing DMEM with 2% FBS

5. Homogenize each sample in media using a new sterile tip for ≈10sec until no large clumps of tissue remain
    a. Note: Hold the homogenizer and tube far back in the hood and tilt the tube away from you to avoid spraying sample out of the hood by accident
6. Remove the homogenizer tip carefully, disassemble the inner blue part from the outer clear part, and place both pieces in a pan of vesphene to disinfect
7. Keep snap-cap tubes with homogenized samples on ice until all samples are finished

ii. Inactivate Virus
1. Add 900μl TriReagent to sterile eppendorf tubes and transfer 100μl of each homogenized tissue to a tube containing TriReagent
2. Vortex all samples very well and let sit at room temperature for 5min with frequent vortexing until no visible clumps of cells are left
3. After incubation, clean out hood, place down a clean hood liner, and transfer sample to a new sterile eppendorf tube
4. Keep the samples at 4°C if you are not going to continue to the next step immediately or store them in -80°C freezer

iii. Extract RNA
1. Turn on Themocycler to 4°C
2. If samples are frozen, thaw to room temperature
3. In BSC, add 200μl chloroform to each tube and invert vigorously for 20sec (DO NOT VORTEX)
4. Let sit at room temperature for 3min
5. Centrifuge at 12,000 x g for 15min at 4°C
6. Transfer 500μl of aqueous phase (clear top layer) to a clean tube
7. The remaining steps can be performed on a benchtop cleaned with RNase Away Reagent; remove tubes from BSC and clean hood with vesphene
8. Add 1 volume (500μl) 70% ethanol to each lysate and mix well by pipetting
9. Transfer up to 700μl of sample at a time to RNeasy Mini Spin column inside a 2ml collection tube (supplied in kit)
10. Centrifuge at >8000 x g for 15sec
11. Discard flow through and repeat with remaining sample
12. Add 350μl Buffer RW1 to spin column
13. Centrifuge at max speed for 30sec and discard flow-through
14. Add 10μl DNase 1 stock solution to 70μl Buffer RDD (Note: This calculation is per reaction. Multiply volumes for more samples.), mix by inverting tube, and briefly centrifuge
15. Add 80μl DNase 1 – Buffer RDD mixture directly to spin column membrane and incubate at room temperature for 15mins

16. Add 500μl Buffer RPE to spin column, centrifuge at max speed for 30sec and discard flow-through
17. Repeat step 16
18. Place spin column in a new collection tube (supplied in kit) and centrifuge at full speed for 1min to dry the membrane
19. Place spin column in a new 1.5ml collection tube with cap (supplied in kit), add 40μl nuclease-free water directly to the spin column membrane, and centrifuge at max speed for 1min to elute RNA
20. Store samples in -80°C freezer

A.3.2 PCR for Viral Titers [30, 40]

1. **Material/Reagents:**
   a. Superscript® III Platinum® One-Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, CA, cat. #11745-100)
   b. MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737)
   c. MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971)
   d. 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA)
   e. RNase/DNase-free microcentrifuge tubes
   f. Nuclease-free water
   g. Swine Influenza A (H1N1) real-time RT-PCR Assay (BEI Resources, NR-15577)
   h. Standard Curve with known virus titers

2. **Preparation of Reagents:**
   a. Nucleic acids extracted from Swine Influenza A (H1N1) Positive Control (NR-15590), and Novel H1N1 2009 (Swine) Influenza A (NR-15627) should be diluted 1:10 prior to use as template in the assay
   b. Working stock aliquots of the primers and probes should be made at concentrations of 40μM (primers) and 10μM (probes) by adding 500μl nuclease-free water to each

Table 5. Influenza PCR Primers and Probes

Primers and Probes	Catalog #	Sequence
*InfA Probe	NR-15578	TGC AGT CCT CGC TCA CTG GGC ACG
InfA Forward	NR-15579	GAC CRA TCC TGT CAC CTC TGA C
InfA Reverse	NR-15580	AGG GCA TTY TGG ACA AAK CGT CTA
**Sw H1 Probe	NR-15584	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A
Sw H1 Forward	NR-15585	GTG CTA TAA ACA CCA GCC TYC CA
Sw H1 Reverse	NR-15586	CGG GAT ATT CCT TAA TCC TGT RGC

*At the 5'end, TaqMan® probes are marked with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3' end with the quencher, Blackhole Quencher 1 (BHQ1) (Biosearch Technologies, Inc., Novato, CA) [41].
**Taqman® probes are labeled at the 5'-end with the reporter molecule 6-carboxyfluorescein (FAM) and quenched internally at a modified "T" residue with BHQ1, with a modified 3'- end to prevent probe extension by Taq polymerase [41].

3. Protocol:

   a. Thaw RNA samples and PCR reagents on ice, keep on ice throughout procedure
   b. Label one microcentrifuge tube for the master mix
   c. All samples, standards and controls should be run in duplicate
   d. Prepare master mix according to table below:

Table 6. PCR Master Mix

Reagent	Volume of Reagent per Reaction
Forward Primer	N x 0.5μl
Reverse Primer	N x 0.5μl
Probe*	N x 0.5μl
Superscript Taq Mix	N x 0.5μl
2X PCR Master Mix	N x 12.5μl
Nuclease-free Water	N x 5.5μl
Total Volume	*N x 20μl*

Note: N = number of samples, controls, and standards, in duplicate; * 6-carboxyfluorescein (6-FAM) probe must be protected from light

   e. After adding water last, mix master mix by pipetting (DO NOT VORTEX)
   f. Add 20μl master mix to all needed wells of a 96-well plate
   g. Add 5μl of each unknown sample, standard or control to each well
      i. Needed controls:
         1. No Template Control (NTC) containing all reagents except an RNA sample, which is replaced with nuclease-free water
         2. Positive Controls containing master mix and known positive virus samples: Swine Influenza A (H1N1) Positive Control (NR-15590) and the Novel H1N1 2009 (Swine) Influenza A Positive Control (NR-15627) should be included in each run for all primer and probe sets
   h. Wearing gloves, smooth a clear adhesive cover over the 96-well PCR plate using the brown tool
   i. Place foam cover on top of clear adhesive cover and take plate to PCR machine on ice
   j. Set up the PCR machine using the parameters in the table below:
      i. Internal control = ROX
      ii. Detector = FAM channel
      iii. Sample volume = 25

Table 7. PCR Machine Parameters for Influenza Detection

Reverse Transcription	50°C for 30 min
Taq Inhibitor Activation	95°C for 2 min
PCR Amplification (40 cycles) Denature/Anneal	95°C for 15 sec 55°C for 30 sec

A.3.3 Making Standard Curve
1. **Material/Reagents:**
   a. Virus stock with a known high titer
      i. A/California/07/2009 (BEI Resources, cat. #NR-13663) pandemic H1N1 virus (original titer: 2.8×10^8 CEID$_{50}$/ml)
      ii. A/California/04/09 (gift provided by Dr. Stacy Schultz-Cherry, St. Jude Children's Research Hospital) pandemic H1N1 virus (original titer: $10^{8.25}$ TCID$_{50}$/ml)
   b. Need all reagents/materials from RNA isolation from liquid samples and PCR protocols above
2. **Protocol:**
   a. Obtain H1N1 virus stock of known titer in TCID$_{50}$/ml
   b. Add 100μl virus stock sample to 900μl TriReagent in six replicates (want a lot of sample so can make a lot of dilutions and aliquot)
   c. Perform RNA extraction procedure for liquid samples and elute RNA in 40μl nuclease-free water
   d. Combine all six replicates together ($40\mu l \times 6 \approx 240\mu l$)
   e. Make 10-fold dilutions with nuclease-free water starting with 1:10 (10^{-1}) and going down to 10^{-8}
   f. Run dilution PCR in duplicate against the remaining standard stock
      i. Note: Make sure to include a NTC
   g. After PCR is complete, check the new standard against the old standard for accuracy
   h. Calculate new standard quantities based off the original known virus titer (TCID$_{50}$/ml)

Table 8. H1N1 Standard Information

Standard	Amy's Standard Quantity	Allison's Standard Quantity
10^{-1}	700000	220000
10^{-2}	70000	22000
10^{-3}	7000	2200
10^{-4}	700	220
10^{-5}	70	22
10^{-6}	7	2.2

A.4 CYTOKINE DETECTION

A.4.1 ELISA
- **Materials/Reagents:**

1. Capture Antibody: Monoclonal Anti-Ferret Interferon Gamma, Clone 4A4B7 (BEI Resources, cat. #NR-4492)
2. Detection Antibody: Monoclonal Anti-Ferret Interferon Gamma, Clone 1H1H12 (BEI Resources, cat. #NR-4493)
3. Secondary Antibody: Goat Anti-Ferret IgG IgA IgM Antibody, peroxidase conjugated (Rockland™ Immunochemicals Inc., Limerick, PA)
4. Ferret serum and blood samples
5. 96-well flab-bottom Immulon 2HB
6. 1X PBS
7. 0.5% Tween-20
8. TM Blue, soluble form
9. 1 N H_2SO_4
10. Blocking Solution: 1% BSA in PBS
11. Multi-pipetter
12. Plate reader PowerWave XS (BioTek) and SpectraMax L

- **Preparation of Reagents (All Procedures):**
    1. 0.05M Bicarbonate Buffer pH 9.6
        Solution A: 5.29 g Na_2CO_3 in 1000 ml dH_2O
        Solution B: 4.20 g $NaHCO_3$ in 1000 ml dH_2O
            *Stir Solution A into Solution B until a pH of 9.6 is obtained
    2. 1% BSA in PBS (Blocking Solution)
            0.5 g BSA
            50 ml of 1X PBS
                *Let stir for at least 10 min; can store for several days at 4°C
    3. 1X PBS/0.05%Tween-20
            500μl Tween-20 in 100ml 1X PBS
                *Use stir-plate to mix solution
    4. TM Blue
            Use undiluted; bring to room temperature before using
    5. 1 N H_2SO_4 Stop Solution
            27.8ml concentrated sulfuric acid (95%-98% pure) in 972.2ml dH20

- **Preparation of Reagents (Procedure #1 Only):**
    6. Antigen
            Use 20μl recombinant ferret IFN-gamma in 880μl 0.05M bicarbonate buffer
    7. Primary Antibody (150μl total volume per antibody type)
            MAb1: 3μl in 147μl blocking solution
                per well, 0.05μl, 0.025μl, 0.0125μl, 0.00625μl
            MAb2: same as MAb1
            Serum (PAb): 15μl in 135μl blocking solution
                Use dilutions 1:10, 1:20, 1:40, 1:80
            Prebleed: 15μl in 135μl blocking solution
                Use dilutions 1:10, 1:20, and two blanks (only blocking solution)
    8. Secondary Antibody
            Use a 1:10,000 dilution: 1μl in 10ml of blocking solution

- **Preparation of Reagents (Procedure #2 Only):**

6. <u>Primary Antibody</u> (Monoclonal Antibody 4A4B7)
   Add 9μl MAb to 891μl bicarbonate buffer
7. <u>Antigen</u>
   Add 3μl recombinant ferret IFN-gamma to 147μl blocking solution
   Serum (PAb): 15μl in 135μl blocking solution
   Use dilutions 1:10 and 1:20
8. <u>Secondary Antibody</u> (Monoclonal Antibody 1H1H12)
   Add 9μl MAb to 891μl blocking solution
9. <u>Anti-ferret -IgG, -IgM -IgA Antibody</u>
   Use dilution 1:10,000 for all wells = 1μl in 9,999μl (10ml) of blocking solution

- **INF-γ ELISA Procedure #1:**
  1. Obtain a 96-well Immulon 2HB flat-bottom ELISA plate and coat 50μl/well with recombinant ferret IFN-g antigen overnight (at least 12-16 hours) at 4°C
  2. Remove the coating solution by dumping the liquid in a sink and patting the plate on a paper towel to remove the remaining liquid
  3. Wash wells twice with 200 μl 1X PBS
  4. Block the wells with 150 μl of 1% BSA in PBS and incubate for one hour at 37°C
  5. Empty wells and blot dry, but **do not wash** them
  6. Dilute the influenza A virus serum (PAb from repository), monoclonal Ab, and prebleed (including two blanks of only blocking solution) from ferrets to its proper amounts and add 50 μl per wells
  7. Wash plate 5 times with PBS/0.5% Tween-20 and blot dry
  8. Add 50 μl/well secondary anti-ferret-IgG, -IgM -IgA antibody (if already peroxidase-labeled) and incubate for one hour at room temperature
  9. Wash wells 5 times with PBS/0.5% Tween-20 and blot dry
  10. Add the substrate, 200 μl of TMB, to each well by putting 4 ml in a conical tube for easier access, and let plate shake for 15-20 minutes at room temperature
  11. Stop color development with 50 μl per well of 1 normal H_2SO_4
  12. Measure the color change using an automated plate reader at the needed specific wavelength, 450nm

- **INF-γ ELISA Procedure #2:**
  1. Obtain a 96-well Immulon 2HB flat-bottom ELISA plate and coat 100μl/well with monoclonal antibody A (4A4B7) overnight (at least 12-16 hours) at 4°C
  2. Remove the coating solution by dumping the liquid in a sink and patting the plate on a paper towel to remove the remaining liquid
  3. Wash wells twice with 200μl 1X PBS
  4. Block the wells with 200μl of 1% BSA in PBS and incubate for one hour at 37°C
  5. Empty wells and blot dry, but **do not wash** them
  6. Dilute the influenza A virus serum (PAb from repository), and recombinant ferret gamma IFN antigen from ferrets to its proper amounts and add 50μl per wells, letting sit for 1 hour at 37°C

7. Wash plate 5 times with 200μl PBS/0.5% Tween-20 and blot dry
8. Add 100μl second monoclonal antibody B (1H1H12) for one hour at 37°C
9. Wash 5 times with 200μl PBS/0.5% Tween-20 and blot dry
10. Add 50μl/well anti-ferret-IgG, -IgM -IgA antibody (if already peroxidase-labeled) and incubate for one hour at room temperature
11. Wash wells 5 times with PBS/0.5% Tween-20 and blot dry
12. Add the substrate, 200μl of TMB, to each well by putting 4 ml in a conical tube for easier access, and let plate shake for 20 minutes at room temperature
13. Stop color development with 50μl/well of 1 normal H_2SO_4
14. Measure the color change using an automated plate reader at the needed specific wavelength, 450nm

- **Calculations for Procedure #1:**
  - *A. Antigen*
    - --Stock = 0.1mg/ml
    - --50μl x 18 wells = 900μl total volume
    - --0.011μg x 18 wells = 0.198 ≈ 0.2μg
    - --0.2μg = 20μl
    - --20μl added to 880μl 0.05M bicarbonate buffer

  - *B. Monoclonal Antibodies*
    - --Stock = 0.1mg/ml = 0.1μg/μl
    - --Top well concentration = 0.05: 1μl MAb in 50μl total volume, so begin with 3μl MAb added to 147μl blocking solution (A)
    - --Concentration 0.025: take 75μl A and add 75μl blocking solution (B)
    - --Concentration 0.0125: take 75μl B and add 75μl blocking solution (C)
    - --Concentration 0.00625: take 75μl C and add 75μl blocking solution (D)
  - *C. PAb (Serum)*
    - --start with a 1:10 dilution = 15μl in 150 total volume = 15μl serum added to 135μl blocking solution (A)
    - --1:20 dilution: take 75μl A and add 75μl blocking solution (B)
    - --1:40 dilution: take 75μl B and add 75μl blocking solution (C)
    - --1:80 dilution: take 75μl C and add 75μl blocking solution (D)
  - *D. Prebleed ferret*
    - --start with a 1:10 dilution = 15μl in 150 total volume = 15μl prebleed added to 135 μl blocking solution (A)
    - --1:20 dilution: take 75μl A and add 75μl blocking solution (B)
- **Calculations for Procedure #2:**
  - *A. Monoclonal Antibody A (4A4B7)*
    - --Stock = 0.1mg/ml = 0.1μg/μl
    - --0.1μg/well x 9 wells = 0.9μg = 9 μl
    - --100μl/well x 9 wells = 900μl total volume
    - --Add 9μl MAb to 891μl bicarbonate buffer
  - *B. Antigen*
    - --Stock = 0.1mg/ml = 0.1μg/μl

--Want 0.1μg/well in 50μl/well

--1μl/well antigen + 49μl/well blocking solution

--0.1μg antigen x 3 wells = 0.3μg = 3 μl antigen

--Add 3μl antigen to 147μl blocking solution

C. PAb (Serum)

--Start with a 1:10 dilution = 15μl in 150 total volume = 15μl serum added to 135μl blocking solution (A)

--1:20 dilution: take 75μl A and add 75μl blocking solution (B)

D. Monoclonal Antibody B (1H1H12)

--Stock = 0.1mg/ml = 0.1μg/μl

--0.1μg/well x 9 wells = 0.9μg = 9μl

--100μl/well x 9 wells = 900μl total volume

--Add 9μl MAb to 891μl blocking solution

A.4.2 PCR

1. RNA Extraction

a. RNeasy® Mini Kit with on-column DNase Digestion

    i. <u>**Materials/Reagents:**</u>

        1. RNeasy® Mini Kit (Qiagen, cat. #774104)

        2. RNase-Free DNase Set (Qiagen, cat. #79254)

        3. Tissue Homogenizer

        4. Eppendorf centrifuge (5415D)

        5. Micropipettes

        6. Ethanol

        7. RNase/DNase-free 1.5ml snap cap tube

        8. Spectrophotometer (NanoDrop®, ND-1000)

    ii. <u>**Protocol:**</u>

        1. Disrupt the tissue (using no more than 30mg) and homogenize the lysate in Buffer RLT. Centrifuge the lysate for 3 min at maximum speed. Carefully pipette out the supernatant into new microcentrifuge tube.

        2. Add 1 volume of 70% ethanol to the lysate and mix well by pipetting (DO NOT CENTRIFUGE).

        3. Transfer up to 700μl of the sample to an RNeasy Mini spin column placed in a 2ml collection tube (supplied by kit). Close the lid and centrifuge for 15 sec at >8000 x g. Discard the flow-through.

        4. Add 350μl Buffer RW1 to the RNeasy spin column, close the lid, and centrifuge for 15 sec at >8000 x g. Discard the flow-though.

        5. Add 10μl DNase I stock solution to 70μl Buffer RDD. Mix by gently inverting tube and centrifuge briefly.

        6. Add 80μl DNase I incubation mix (made above, step 5) directly to the spin column membrane and let sit on bench top for 15 mins (20-30°C).

        7. Add 350μl Buffer RW1 to the RNeasy spin column, close the lid, and centrifuge for 15 sec at >8000 x g. Discard the flow-though.

8. Add 500μl Buffer RPE to RNeasy spin column, close the lid, and centrifuge for 15 sec at >8000 x g. Discard the flow-though.
9. Repeat Step 8.
10. Place RNeasy spin column in a new 2ml collection tube (supplied by kit) and centrifuge for 1 min at max speed to dry the membrane.
11. Place the RNeasy spin column inside a new 1.5ml snap cap tube (supplied). Add 30-50μl RNase-free water directly to the spin column membrane, close the lid, and centrifuge for 1 min at max speed to elute the RNA.
12. If the expected yield of RNA is >30μg, repeat steps 8-11 using another 30-50μl RNase-fee water. If a high RNA concentration is required, use elute from step 7 to repeat through step 11.
13. Nanodrop for RNA purity and concentration.

b. Invitrogen PureLink Viral RNA/DNA Kit
   i. **Materials/Reagents:**
      1. PureLink Viral RNA/DNA Kit (Invitrogen, cat. #12280-050)
      2. 15ml conical tube
      3. 96-100% ethanol
      4. Nuclease-free microcentrifuge tubes
      5. Biological Safety Cabinet II (BSC)
      6. RNase Away® Reagent (Life Technologies, Thermo Scientific, cat. #10328011)
      7. -20°C and -80°C freezers
   ii. **Preparation of reagents:**
      1. Add 60ml 96-100% ethanol to wash buffer in kit
      2. Add 310μl nuclease-free water to carrier RNA (this makes a 1μg/μl solution), mix, aliquot, and store at -20°C
   iii. **Protocol:**
      1. Prepare lysis buffer with carrier RNA
         a. N x 0.21ml (volume lysis buffer/reaction) = A ml lysis buffer
         b. A ml x 28μl//ml = B μl carrier RNA stock solution
         c. In a sterile 15ml conical tube add calculated volumes of carrier stock solution (B) to the lysis buffer (A) and mix by pipetting (do not vortex)
         d. Store solution at 4°C and use buffer within 1 hour
      2. Let frozen samples thaw on ice
      3. Add 25μl Proteinase K in a sterile microcentrifuge tube
      4. Add 200μl RNA sample into the tube containing the Proteinase K
      5. Inside BSC, add 200μl lysis buffer with carrier RNA
      6. Vortex all samples for 15sec, then the rest of the procedure can be performed on the benchtop
      7. Incubate samples in 56°C water bath for 15mins
      8. Add 250μl 96-100% ethanol to all samples, vortex, and let sit at room temperature for 5mins

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9. Transfer the lysate to the viral spin column in a collection tube (supplied) (viral spin column can hold 700μl volume max)
10. Centrifuge at 6800 x g for 1min
11. Discard the collection tube and place spin column in a new collection tube (supplied)
12. Add 500μl wash buffer with ethanol (prepared above, supplied by kit) to all viral spin columns
13. Centrifuge at 6800 x g for 1min and discard the flow through
14. Repeat step 11-12
15. Discard collection tube and place viral spin column in new collection tube
16. Centrifuge at max speed for 1 min to dry the viral spin column
17. Place the viral spin column inside a clean Eppendorf tube (supplied by kit)
18. Elute RNA with 40μl sterile RNase-free water (supplied) directly to the viral spin column cartridge
19. Incubate samples at room temperature for 1min
20. Centrifuge at max speed for 1min
21. Discard the viral spin column and store the purified viral RNA at −80°C

c. QIAamp® Viral RNA Mini Kit
   i. **Materials/Reagents:**
      1. QIAamp® Viral RNA Mini Kit (Qiagen, cat. #52904)
      2. 15ml conical tube
      3. 96-100% ethanol
      4. 1.5ml nuclease-free microcentrifuge tubes
      5. Biological Safety Cabinet II (BSC)
      6. RNase Away Reagent
      7. -20°C and -80°C freezers
      8. Nasal wash samples
      9. 80°C incubator
   ii. **Preparation of reagents:**
      1. Add 25ml 96-100% ethanol to Buffer AW1 in kit (44ml total volume)
      2. Add 30ml 96-100% ethanol to Buffer AW2 in kit (43ml total volume)
      3. Add 310μl Buffer AVE to carrier RNA (this makes a 1μg/μl solution), mix, aliquot, and store at -20°C (do not freeze-thaw the aliquots more than 3 times)
      4. Check Buffer AVL for precipitate and if needed, incubate at 80°C until dissolved
   iii. **Protocol:**
      1. Prepare Buffer AVL with carrier RNA
         a. N x 0.56ml (volume Buffer AVL/reaction) = A ml Buffer AVL
         b. A ml x 10μl/ml = B μl carrier RNA

     c. In a sterile 15ml conical tube add calculated volumes of carrier stock solution (B) to Buffer AVL (A) and mix by inverting tube 10 times (do not vortex)

     d. Buffer AVL with carrier RNA should be prepared fresh

     e. This solution is stable at 2-8°C for up to 48 hours, but will develop a precipitate that must be dissolved at 80°C for ≤5min before use (frequent warming and extended incubation can cause degradation of carrier RNA leading to false-negative results, especially in low titer samples)

2. Add 560μl Buffer AVL with carrier RNA into sterile microcentrifuge tube

     a. For sample volumes >140μl increase the amount of Buffer AVL with carrier RNA proportionally and use larger tubes (e.g. 280μl sample requires 1120μl Buffer AVL with carrier RNA)

3. Add 140μl sample to each tube and mix by vortexing for 15sec

4. Incubate at room temperature for 10mins and centrifuge briefly to remove drops from inside the lid

5. Add 560μl 96-100% ethanol to the sample, mix by pulse-vortexing for 15sec, and centrifuge briefly

     a. For sample volumes >140μl, increased amount of ethanol proportionally (e.g. 280μl sample requires 1120μl ethanol)

6. Add 630μl solution from step 5 to the QIAamp Mini column in a collection tube (supplied)

7. Centrifuge at 6000 x g (8000rpm) for 1min

8. Place mini column in a new collection tube and discard the old collection tube

     a. If the solution has not completely passed through the membrane, centrifuge again at a higher speed

9. Repeat steps 6-8 until all solution has passed through column membrane

10. Add 500μl Buffer AW1 to each sample

     a. Do not need to increase this volume for samples >140μl

11. Centrifuge at 6000 x g (8000rpm) for 1 min

12. Place mini column in a new collection tube and discard the old tube

13. Add 500μl Buffer AW2 to each sample

14. Centrifuge at full speed for 3mins

15. Dump flow-through and centrifuge at full speed for 1min to dry column

16. Place mini column in a clean 1.5ml microcentrifuge tube, add 60μl Buffer AVE to each sample

17. Incubate at room temperature for 1min

18. Centrifuge at 6000 x g (8000rpm) for 1min to elute RNA

19. Store extracted sample at -80°C for up to 1 year

2. cDNA Synthesis for semi-quantitative RT-PCR

   a. Maxima First Strand cDNA Synthesis Kit

      i. Materials/Reagents:

         1. Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, cat. #K1642)

         2. PCR machine (MJ Research, Peltier Thermal Cycler (PCT)-200)

         3. Eppendorf Centrifuge (5415D)

         4. RNase/DNase-free 1.5ml snap cap tubes

         5. RNase/DNase-free 0.2ml omnitubes with flat cap (Thermo Scientific, cat. #HBTC6202N)

      ii. Protocol:

         1. Thaw (on ice), mix and briefly centrifuge the components of the kit, storing them on ice for the duration of the procedure.

         2. Into a sterile 1.5ml tube on ice, add:

Table 9. cDNA Master Mix

Reagent	Volume
5X Reaction Mix	4μl
Maxima Enzyme Mix	2μl
Template RNA	1μg
Nuclease-free water	? μl (adjusted to make total volume 20μl)
Total Volume	*20μl*

         3. Mix gently, centrifuge, and incubate for 10 mins at 25°C followed by 15 mins at 50°C (reaction) and terminate by heating for 5 mins at 85°C.

            a. Note: For RNA templates greater than 1μg, prolong the reaction time to 30 mins. For GC-rich RNA templates, or templates that have a large amount of secondary structure, increase the reaction temperature to 65°C.

         4. The cDNA product can be used directly in qPCR or stored at −80°C. Avoid freeze/thaw cycles of cDNA.

         5. Controls needed to verify the results of of the first strand cDNA synthesis:

            a. Reverse Transcriptase minus (RT-) negative control should contain every reagent for the reverse transcription reaction except for the Maxima Enzyme Mix. This control will help assess genomic DNA contamination of the RNA sample.

            b. No Template Control (NTC) should contain every reagent for the reverse transcription reaction except the RNA template, which should be replaced with Nuclease-free water. This control will help assess reagent contamination.

   b. Maxima First Strand cDNA Synthesis with dsDNase Kit

      i. **Materials/Reagents:**

1. Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (Thermo Scientific, cat. #K1672)
2. PCR machine (MJ Research, Peltier Thermal Cycler (PCT)-200)
3. Eppendorf centrifuge (5415D)
4. RNase/DNase-free 1.5ml snap cap tubes
5. RNase/DNase-free 0.2ml omnitubes with flat cap (Thermo Scientific, cat. #HBTC6202N)
6. 37°C water bath

ii. Protocol:

1. Thaw (on ice), mix and briefly centrifuge the components of the kit, storing them on ice for the duration of the procedure
2. Into a sterile 0.2ml tube on ice, add:

Table 10. Addition of dsDNase to RNA Template

Reagent	Volume
10X dsDNase Buffer	1µl
dsDNase	1µl
Template RNA	1pg-5µg
Nuclease-free water	? µl (adjusted to make total volume 10µl)
Total Volume	*10µl*

3. Mix gently, centrifuge, and incubate for 2 mins in 37°C water bath
4. Chill on ice, briefly centrifuge, and place on ice
5. Add the following to the same tube:

Table 11. Addition of cDNA Components to RNA Template

Reagent	Volume
5X Reaction Mix	4µl
Maxima Enzyme Mix	2µl
Nuclease-free water	? µl
Total Volume	*10µl*

6. Gently mix and centrifuge
7. In a PCR machine, incubate at 25°C for 10min, 50°C for 15min (reaction) and terminate at 85°C for 5min
   a. For RNA templates >1µg increase reaction time to 30min
   b. For GC-rich RNA templates or templates with large amount secondary structure increase reaction temp to 65°C
8. The cDNA product can be used directly in qPCR or stored at −80°C. Avoid freeze/thaw cycles of cDNA.
9. Controls needed to verify the results of the first strand cDNA synthesis:
   a. Reverse Transcriptase minus (RT-) negative control should contain every reagent for the reverse transcription reaction

except Maxima Enzyme Mix, which is replaced with nuclease-free water. This control will help assess genomic DNA contamination of the RNA sample.
   b. No Template Control (NTC) should contain every reagent for the reverse transcription reaction except the RNA template, which should be replaced with Nuclease-free water. This control will help assess reagent contamination.

3. **PCR:** Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific, cat. #K0221) [26, 42]

   a. **Materials/Reagents:**
      i. Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific, cat. # K0221)
      ii. RNase Away (Life Technologies, Thermo Scientific, cat. #10328011)
      iii. MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems™, Life Technologies, Thermo Scientific, cat. #4306737)
      iv. MicroAmp™ Optical Adhesive Film (Applied Biosystems™, Thermo Scientific, cat. #4311971)
      v. Primers: IFN-γ, TNF-α, IL-10, GAPDH (Integrated DNA Technologies, Coralville, IA)

Table 12. Cytokine Ferret Primers

Primers	Sequence (5'>3')
IFN-γ Forward	CCATCAAGGAAGACATGCTTGTCAGG
IFN-γ Reverse	CTGGACCTGCAGATCATTCACAGGAA
TNF-α Forward	TGGAGCTGACAGACAACCAGCTAA
TNF-α Reverse	TGATGGTGTGGGTAAGGAGCACAT
IL-10 Forward	TCCTTGCTGGAGGACTTTAAGGGT
IL-10 Reverse	TCCACCGCCTTGCTCTTATTCTCA
GAPDH Forward	AACA TCA TCCCTGCTTCCACTGGT
GAPDH Reverse	TGTTGAAGTCGCAGGAGACAACCT

   b. **Protocol:**
      iv. Thaw (on ice), gently vortex and briefly centrifuge all reagents
      v. Prepare a PCR reaction master mix by adding the following components (except template DNA) into a sterile snap cap tube at room temperature:

Table 13. PCR Reagents

Reagent	Volume of Reagent Added per Reaction
Maxima SYBR Green 2X	12.5μl
Forward Primer	2μl (0.8μM)
Reverse Primer	2μl (0.8μM)
Nuclease-Free Water	Up to 25μl
Template cDNA	≤500ng
Total Volume	*N x 25μl*

Note: PCR reaction mix is made for each primer set. N = number of samples/ reactions, including controls.

    vi. Needed Controls:
        1. No Template Control (NTC) containing all components except template DNA, which is replaced with Nuclease-free water. This control is important to assess reagent contamination or primer degradation.
        2. Reverse Transcriptase Minus (RT-) control containing all components except Maxima SYBR Green (2x). This control is important for assessing RNA sample contamination with genomic DNA.
    vii. Mix the PCR reaction mix and dispense into the needed wells of a PCR plate
    viii. Add Template DNA (≤500ng/reaction) to the appropriate PCR plate wells containing the master mix
        1. Note: For two-step RT-qPCR, the volume of cDNA added from the RT reaction should not exceed 10% of the final PCR volume
    ix. Gently mix the reactions in the wells (DO NOT VORTEX) without creating bubbles, as these will interfere with fluorescence detection, and centrifuge briefly if needed
    x. Program the thermal cycler according to the instructions below, place the PCR plate inside the machine, and start the program

Table 14. Gene Expression Program

Optional: UDG pre-treatment	50°C for 2 min
Initial Denaturation	95°C for 10 min
PCR Amplification (40 cycles)	95°C for 15 sec 60°C for 30 sec 72°C for 30 sec

Note: SYBR Green (detector) data should be collected during the 72°C extension step

    xi. Expression levels should be normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and calculated as fold change compared to day 0 ferrets based on the ΔΔCt method

xii. The ΔCt is calculated by subtracting the gene of interest at each time point from the GAPDH Ct value. Then fold change is calculated by subtracting the day 0 ΔCt values from the subsequent days ΔCt values. The formula: fold change $= 2^{-\Delta\Delta Ct}$ is then used to obtain absolute values [31]

A.4.3 Blood Processing

1. **Materials/Reagents:**
   a. Blood sample
   b. Lymphoprep™ (Stemcell, cat. #07861)
   c. 50ml SepMate™ tube (Stemcell, cat. #15470)
   d. 50mL conical tube (Fisher, cat. #1443222)
   e. PBS + 2% FBS
      i. Dulbecco's Phosphate Buffered Saline (D-PBS) (Fisher, cat. #SH3025801)
      ii. Fetal Bovine Serum (Atlanta Biological, Cat. #S11150)
   f. Sorvall Legend RT+ Centrifuge (Thermo Scientific, cat. #75004377)
   g. Trypan Blue (Fisher, cat. #SV3008401)
   h. Hemacytometer
   i. Microscope (Olympus CKX41)
   j. 2mL Cryovials (Fisher, cat. #033377Y)
   k. Serological Pipettes (Fisher, cat. #1367522, 1367520, 136682)
   l. Biohazard Waste Container
   m. Sharpie
   n. Cell Freezing Medium (90% FBS/10% DMSO)
      i. Dimethyl Sulphoxide (DMSO) (Sigma, cat. #D2650)
   o. RNA*later*® Solution (Ambion, cat. #AM7021)
   p. Cryopreservation container
   q. Water bath
   r. Liquid nitrogen storage
   s. Vesphene

2. **Protocol:**
   a. Bring samples, PBS + 2% FBS and Lymphoprep™ to room temperature and mix each reagent by inverting
   b. Add 15mL of Lymphoprep™ to SepMate™-50mL tubes by pipetting it through the center hole of the SepMate ™ inserts. Set tube aside.
   c. Gently mix blood sample by inverting up and down. Pour blood sample into 50mL conical tube and determine combined volume.
   d. Dilute blood sample with equal volume of PBS + 2% FBS, washing out the original blood tubes. Discard blood sample tubes in bleach waste container. Mix 50ml conical tube containing blood and PBS/FBS gently by inverting.
   e. Add the diluted sample by pipetting it down the side of the SepMate™ tube. The sample will mix with the Lymphoprep™ above the insert. Rinse out the conical tube with bleach and discard.
   f. Centrifuge the SepMate™ tube at 1200 X g for 10 minutes at room temperature, with the brake on.
      i. Note: For samples that sit 4 to >24 hours, a centrifugation time of 20 minutes is recommended.

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g. Remove 1-1.5mL aliquots of plasma into 2ml cryovials. Plasma samples should be stored at −80°C.

h. Quickly pour the buffy coat and remaining plasma (clear top layer) into a new 50mL conical tube; pouring should be 2 seconds or less being careful not to allow blood to come up through middle hole in bottom of tube. Discard the SepMate™ tube.

i. Wash enriched MNCs with PBS + 2% FBS by filling the conical tube up to the 50mL line. Centrifuge at 500 X g for 10 minutes at room temperature, with the brake on (wash 1).

j. Discard supernatant, repeat wash by resuspending pellet in 50mL PBS + 2% FBS and centrifuge at 500 X g for 10 minutes at room temperature, with the brake on (wash 2).

k. Discard supernatant and resuspend the pellet with 10mL PBS + 2 % FBS and remove 5uL for a 1:5 dilution (shown below).

l. For a 1:5 dilution, add 20uL Trypan Blue and 5uL sample for a total volume of 25uL into one well of a 96-well plate. Remove 10uL of mixture to put in Hemocytometer. Count total white blood cells in the four outer chambers and divide by four to get an average number of cells (#). This number is 10^4 cells/mL. Since the dilution is 1:5, it is # X 10^4 X 5 cells/mL. Then to calculate the total number of cells, multiple these numbers by the total volume of the cell suspension (10mls). So, # X 10^4 X 5 X 10 = cells total in current 10ml volume.

    i. To count cells in a 1:2 dilution, add 10uL sample to 10uL Trypan blue in one well of a 96-well plate and pipette to mix. Remove 10uL of mixture to put in Hemocytometer. Count all four quadrants (16 squares each), add them together, then divide by 4 to get a #. This number is 10^4 cells/mL. Since the dilution is 1:2, it is # X 10^4 X 2 cells/mL. Then to calculate the total number of cells, multiple these numbers by the total volume of the cell suspension (# X 10^4 X 2 X 10 cells total).

m. Bring 10ml MNCs up to 50 ml with PBS + 2% FBS by filling the conical tube up to the 50mL line. Centrifuge at 500 X g for 10 minutes at room temperature, with the brake on (wash3).

n. Discard supernatant and resuspend pellet with 1mL cell-freezing media per 5 x 10^6 to 1 X 10^7 cells/mL (5-10 million cells/mL) based on cell counts.

o. Dispense 1mL per cryotube and place in cryopreservation container (Mr. Frosty) at -80°C allowing slow freeze. Cells should be kept a minimum of 24 hours and a maximum of 72 hours at -80°C.

p. Transfer vials for long-term storage into liquid nitrogen tank.

APPENDIX B: SUPPLEMENTAL RESULTS

B.1   STUDY #2

Table 15. Inhaled Dose of Influenza for Each Ferret from Study #2

Run	Ferret ID	Treatment	Inhaled Dose (TCID$_{50}$)
1	F14-007	Control	834.50
1	F14-008	Control	760.60
1	F14-009	Control	775.50
1	F14-010	Control	819.90
Average Run 1			797.63
2	F14-011	2.5mg/kg −2hr	1102.70
2	F14-012	2.5mg/kg −2hr	1047.10
2	F14-015	2.5mg/kg −2hr	1047.10
2	F14-016	2.5mg/kg −2hr	1091.70
Average Run 2			1072.15
3	F14-013	10mg/kg −2hr	797.80
3	F14-014	10mg/kg −2hr	753.10
3	F14-017	10mg/kg −2hr	730.40
3	F14-018	10mg/kg −2hr	783.00
Average Run 3			766.08
Overall Average Inhaled Dose:			878.62

*Note: F14-018 died on day 2 of study #2 and was not included in data analysis

Table 16. Temperature (°C) of Ferrets in Study #2

Day	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
0	38.7	38.7	38.7	38.6	39.0	37.9	38.2	38.4	39.0	39.0	38.8
1	38.9	38.3	38.6	38.7	38.7	38.5	38.9	38.7	38.7	38.7	39.4
2	40.0	40.2	40.1	40.6	39.8	40.7	40.5	40.9	39.8	39.8	39.9
3	38.5	39.4	38.7	38.1	39.9	38.9	39.3	39.0	39.9	39.9	39.8

Table 17. Weight (kg) of Ferrets in Study #2

Day	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
0	0.86	0.76	0.78	0.84	0.75	0.7	0.7	0.74	0.81	0.75	0.72
1	0.87	0.77	0.78	0.84	0.75	0.67	0.72	0.74	0.86	0.75	0.71
2	0.86	0.74	0.75	0.84	0.68	0.65	0.69	0.72	0.85	0.68	0.71
3	0.86	0.71	0.75	0.84	0.69	0.64	0.68	0.7	0.83	0.69	0.7

Table 18. Weight Change (%) of Ferrets in Study #2

Day	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	1.16	1.32	0.00	0.00	0.00	-4.29	2.86	0.00	6.17	0.00	-1.39
2	0.00	-2.63	-3.85	0.00	-9.33	-7.14	-1.43	-2.70	4.94	-9.33	-1.39
3	0.00	-6.58	-3.85	0.00	-8.00	-8.57	-2.86	-5.41	2.47	-8.00	-2.78

Table 19. Clinical Scores of Ferrets in Study #2

Day	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	4	4	0	0	2	2	0	0	0
2	34	38	24	24	21	21	19	24	0	21	2
3	56	50	46	46	29	29	21	26	26	31	4

Table 20. Nasal Wash Viral Titers (CEID$_{50}$/ml Equivalent) for Study #2

	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
Day	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
1	5.99E+02	5.92E+04	1.51E+04	3.14E-01	1.43E+03	5.33E+03	1.30E+03	6.50E+00	1.77E+01	4.41E+01	5.83E+02
2	1.61E+06	6.66E+05	4.16E+05	4.04E+05	8.73E+05	3.48E+05	3.42E+03	3.77E+05	8.65E+01	2.23E+05	1.20E+05
3	2.97E+04	1.49E+04	1.76E+03	4.05E+03	6.33E+04	3.85E+03	8.02E+04	4.69E+04	3.16E+05	1.29E+05	1.87E+04

Table 21. Lung 4/6 Viral Titers (CEID$_{50}$/ml Equivalent) for Study #2

	F14-007	F14-008	F14-009	F14-010	F14-011	F14-012	F14-015	F14-016	F14-013	F14-014	F14-017
Day	Control				2.5mg/kg HB36.6 -2hr				10mg/kg HB36.6 -2hr		
3	3.35E+05	6.38E+05	5.21E+04	3.68E+04	6.43E+02	1.58E+03	1.27E+04	4.40E+02	4.82E+05	6.54E+04	4.55E+04

B.2   STUDY #3

Table 22. Inhaled Dose of Influenza for Each Ferret from Study #3

Run	Ferret ID	Treatment	Inhaled Dose (TCID$_{50}$)
1	F15-003	Control	1700.50
1	F15-006	20mg/kg -24hrs	1556.70
1	F15-007	20mg/kg -24hrs	1474.90
Average Run 1			1577.37
2	F15-004	Control	2021.90
2	F15-008	20mg/kg -24hrs	2000.50
2	F15-009	20mg/kg -24hrs	2021.90
Average Run 2			2014.77
3	F15-001	20mg/kg -2hrs	1524.20
3	F15-002	20mg/kg -2hrs	1491.40
3	F15-005	10mg/kg -2hrs	1605.10
Average Run 3			1540.23
Overall Average Inhaled Dose:			1710.79

Table 23. Temperature (°C) of Ferrets in Study #3

Day	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
0	38.6	37.2	38.6	39.4	38.1	38.1	38.5	38.6
1	39.7	38.7	39.3	39.9	39.1	39.3	38.7	39.6
2	39.8	38.1	40.2	39.4	40.4	39.6	39.2	40.0
3	38.0	37.9	38.9	39.9	40.1	39.5	38.7	Missing Data
4	37.9	38.4	38.4	39.4	39.4	39.1	39.3	37.8
5	37.6	38.4	39.8	38.2	40.1	40.8	38.8	38.3
6	39.9	38.2	37.9	39.6	38.3	39.3	38.3	37.9

Table 24. Weight (kg) of Ferrets in Study #3

Day	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
0	0.81	0.71	0.70	0.68	0.72	0.67	0.70	0.71
1	0.79	0.68	0.72	0.70	0.73	0.65	0.68	0.71
2	0.72	0.62	0.65	0.63	0.63	0.57	0.62	0.63
3	0.72	0.50	0.65	0.60	0.62	0.53	0.59	0.63
4	0.71	0.59	0.63	0.65	0.65	0.58	0.62	0.61
5	0.70	0.58	0.64	0.62	0.64	0.56	0.60	0.61
6	0.68	0.55	0.63	0.59	0.61	0.54	0.58	0.59

Table 25. Change in Weight (%) of Ferrets in Study #3

Day	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	-2.47	-4.23	2.86	2.94	1.39	-2.99	-2.86	0.00
2	-11.11	-12.68	-7.14	-7.35	-12.50	-14.93	-11.43	-11.27
3	-11.11	-29.58	-7.14	-11.76	-13.89	-20.90	-15.71	-11.27
4	-12.35	-16.90	-10.00	-4.41	-9.72	-13.43	-11.43	-14.08
5	-13.58	-18.31	-8.57	-8.82	-11.11	-16.42	-14.29	-14.08
6	-16.05	-22.54	-10.00	-13.24	-15.28	-19.40	-17.14	-16.90

Table 26. Clinical Scores of Ferrets in Study #3

	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
Day	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	4	7	6	13	2	4	4	4
3	18	18	18	18	16	14	27	18
4	36	38	33	31	21	26	16	22
5	27	37	19	14	15	13	11	11
6	30	38	24	9	11	13	11	16

Table 27. Nasal Wash Viral Titers (CEID$_{50}$/ml or TCID$_{50}$/ml Equivalent) for Study #3

	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
Day	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
1	6.83E+02	4.63E+03	2.43E+03	1.27E+03	2.46E+03	2.74E+03	2.67E+03	3.75E+03
2	3.04E+03	1.35E+04	2.95E+04	1.35E+04	2.85E+04	5.28E+03	3.18E+03	6.56E+03
3	4.23E+03	5.26E+04	5.46E+03	1.31E+05	2.08E+04	1.33E+04	1.05E+04	5.84E+04
4	8.55E+02	3.71E+03	1.27E+02	1.19E+03	9.97E+02	1.01E+03	4.23E+02	1.99E+03
5	5.00E+03	2.76E+03	5.12E+03	1.92E+04	3.07E+04	1.30E+04	2.95E+03	5.14E+03
6	8.15E+01	3.04E+04	7.68E+03	7.91E+02	2.51E+02	4.71E+03	2.13E+02	1.16E+02

Table 28. Lung 4/6 Viral Titers (TCID$_{50}$/ml Equivalent) for Study #3

	F15-003	F15-004	F15-001	F15-002	F15-006	F15-007	F15-008	F15-009
Day	Control		20mg/kg HB36.6 -2hr		20mg/kg HB36.6 -24hr			
6	4.08E+02	2.66E+03	1.95E+04	1.55E+03	6.37E+04	3.71E+03	7.67E+03	2.22E+06

Table 29. Inhaled Dose of Influenza for Each Ferret from Study #4

Run	Ferret ID	Treatment	Inhaled Dose (TCID$_{50}$)
1	F15-010	Control	2115.30
1	F15-012	20mg/kg D1-5	2220.20
1	F15-013	Tamiflu	2505.60
Average Run 1			2280.37
2	F15-011	Control	4048.10
2	F15-016	Tamiflu	3687.30
2	F15-019	20mg/kg D1-5	3451.50
Average Run 2			3728.97
3	F15-014	20mg/kg −2hr	3852.70
3	F15-015	20mg/kg −2hr	3687.30
3	F15-018	Tamiflu	3918.20
3	F15-020	20mg/kg D1-5	3348.80
Average Run 3			3701.75
4	F15-017	Tamiflu	10401.10
4	F15-021	20mg/kg D1-5	8906.80
4	F15-022	Control	10486.70
Average Run 4			9931.53
Overall Average Inhaled Dose:			4817.66

Table 30. Temperature (°C) of Ferrets in Study #4

	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
Day	**Control**	**20mg/kg HB36.6 -2hr**		**20 mg/kg HB36.6 D1-D5**				**5mg/kg Tamiflu D1-D5**			
0	38.5	38.2	38.6	38.4	39.3	37.7	39.0	38.9	38.8	38.7	38.1
1	38.6	38.1	38.6	39.0	37.3	38.4	37.7	39.8	38.8	39.2	38.8
2	40.1	39.9	40.1	39.1	40.4	38.9	38.8	40.3	39.4	39.7	38.8
3	39.7	39.4	39.1	37.4	37.9	37.6	37.6	37.3	38.3	38.3	38.8
4	38.3	39.4	38.9	37.0	37.4	37.5	37.0	39.0	38.3	38.0	38.2
5	39.7	38.8	39.1	39.6	39.3	39.5	38.1	39.2	37.7	38.7	39.2
6	40.3	39.0	39.2	38.1	37.5	37.7	37.8	39.3	39.4	38.8	38.5
7	38.4			38.7	38.4				38.7	38.4	

*Blank spaces on day 7 indicate ferret was necropsied on day 6.

Table 31. Weight (kg) of Ferrets in Study #4

Day	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
	Control	20mg/kg HB36.6 -2hr		20 mg/kg HB36.6 D1-D5				5mg/kg Tamiflu D1-D5			
0	0.92	0.88	0.83	0.80	0.76	0.73	0.74	0.94	0.83	0.91	0.90
1	0.91	0.88	0.80	0.79	0.74	0.71	0.73	0.93	0.81	0.88	0.88
2	0.84	0.82	0.77	0.74	0.69	0.66	0.67	0.88	0.76	0.83	0.84
3	0.81	0.73	0.78	0.71	0.67	0.63	0.64	0.85	0.74	0.80	0.82
4	0.78	0.75	0.71	0.69	0.65	0.61	0.62	0.84	0.78	0.77	0.82
5	0.80	0.69	0.74	0.68	0.64	0.61	0.59	0.82	0.75	0.83	0.82
6	0.79	0.71	0.68	0.67	0.63	0.58	0.60	0.79	0.77	0.81	0.78
7	0.80			0.66	0.62				0.75	0.83	

*Blank spaces on day 7 indicate ferret was necropsied on day 6.

Table 32. Weight Change (%) of Ferrets in Study #4

Day	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
	Control	20mg/kg HB36.6 -2hr		20 mg/kg HB36.6 D1-D5				5mg/kg Tamiflu D1-D5			
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	-1.09	0.00	-3.61	-1.25	-2.63	-2.74	-1.35	-1.06	-2.41	-3.30	-2.22
2	-8.70	-6.82	-7.23	-7.50	-9.21	-9.59	-9.46	-6.38	-8.43	-8.79	-6.67
3	-11.96	-17.05	-6.02	-11.25	-11.84	-13.70	-13.51	-9.57	-10.84	-12.09	-8.89
4	-15.22	-14.77	-14.46	-13.75	-14.47	-16.44	-16.22	-10.64	-6.02	-15.38	-8.89
5	-13.04	-21.59	-10.84	-15.00	-15.79	-16.44	-20.27	-12.77	-9.64	-8.79	-8.89
6	-14.13	-19.32	-18.07	-16.25	-17.11	-20.55	-18.92	-15.96	-7.23	-10.99	-13.33
7	-13.04			-17.50	-18.42				-9.64	-8.79	

*Blank spaces on day 7 indicate ferret was necropsied on day 6.

Table 33. Clinical Scores of Ferrets in Study #4

Day	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
	Control	20mg/kg HB36.6 -2hr		20 mg/kg HB36.6 D1-D5				5mg/kg Tamiflu D1-D5			
0	0	0	0	0	0	0	0	0	0	0	0
1	2	0	0	2	2	0	0	2	0	0	2
2	38	18	28	31	41	26	48	21	33	31	38
3	21	18	23	36	51	33	41	26	26	21	26
4	21	23	16	31	31	46	33	28	28	28	18
5	17	28	23	23	25	48	33	21	14	7	21
6	21	26	24	34	36	43	43	21	7	2	26
7	14			26	18				4	4	

*Blank spaces on day 7 indicate ferret was necropsied on day 6.

Table 34. Nasal Wash Viral Titers ($CEID_{50}$/ml Equivalent) for Study #4

	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
Day	Control	20mg/kg HB36.6 -2hr		20 mg/kg HB36.6 D1-D5				5mg/kg Tamiflu D1-D5			
3	1.77E+06	1.65E+06	3.08E+05	3.03E+05	9.55E+05	6.17E+05	1.65E+06	6.54E+05	2.02E+06	6.26E+05	2.35E+06
6	3.68E+05	3.76E+05	6.79E+05	3.01E+05	2.43E+05	9.37E+05	5.31E+05	7.37E+05	1.01E+06	3.33E+05	4.11E+05

Table 35. Lung 4/6 Viral Titers ($TCID_{50}$/ml Equivalent) for Study #4

	F15-022	F15-014	F15-015	F15-012	F15-019	F15-020	F15-021	F15-013	F15-016	F15-017	F15-018
Day	Control	20mg/kg HB36.6 -2hr		20 mg/kg HB36.6 D1-D5				5mg/kg Tamiflu D1-D5			
6	1.25E+01	3.97E+04	1.61E+04	3.75E+02	8.79E+02	5.91E+03	2.59E+04	2.39E+03	6.44E+02	3.41E+02	2.83E+04

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