Summer 8-28-2020

The Kinetics Of Influenza A (A/CALIFORNIA/4/2009-H1N1) And B (B/PHUKET/3073/2013 (BYAM), B/BRISBANE/60/2008 (BVIC)) Virus Infection In Ferrets

Demi L. Erickson
University of South Dakota

THE KINETICS OF INFLUENZA A (A/CALIFORNIA/4/2009-H1N1) AND B (B/PHUKET/3073/2013 (BYAM), B/BRISBANE/60/2008 (BVIC)) VIRUS INFECTION IN FERRETS

by

Demi L. Erickson

A Thesis Submitted in Partial Fulfillment
Of the Requirements for the
University Honor's Program

Department of Biology
The University of South Dakota
May 2020

The members of the Honors Thesis Committee appointed
to examine the thesis of Demi L. Erickson
find it satisfactory and recommend that it be accepted.

Dr. Victor Huber
Associate Professor of Basic Biomedical Sciences
Director of the Thesis Committee

Dr. Karen Koster
Professor of Biology

Beate Wone
Instructor of Biology

ABSTRACT

The Kinetics of Influenza A (A/California/4/2009-H1N1) and B (B/Phuket/3073/2013 (BYam), B/Brisbane/60/2008 (BVic)) Virus Infection in Ferrets

Demi L. Erickson

Thesis Director: Victor C. Huber, Ph.D.

Influenza is a persistent threat to individual and global health. Seasonal influenza kills nearly 500,000 people globally each year. Influenza viruses have circulated within the human population, causing a significant disease burden. In order to create the most effective antiviral therapies, knowledge concerning all lineages of influenza must be studied accurately and thoroughly. The influenza virus itself is not always responsible for these mortalities, rather, the secondary bacterial infections associated with death. In order to protect the population and examine the secondary infections that arise, the kinetics of influenza must be studied entirely. To understand the kinetics, researchers are turning to the use of laboratory experimentation using ferrets as an effective model. This is done through the use of common laboratory procedures such as $TCID_{50}$'s and hemagglutination assays. Through the use of these procedures, the kinetics of influenza A and B can be confirmed. Within this study, it was confirmed that influenza A infects both the upper and lower respiratory system, while influenza B infects the upper respiratory tract but fails to reach the lower lungs. Understanding the kinetics of influenza is the first step toward creating effective therapies to reduce the mortality and illness associated with the influenza virus infection.

Keywords: Influenza, Ferrets, Kinetics, $TCID_{50}$

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I would like to express my deepest and most sincere gratitude to my research director, Dr. Victor Huber, for giving me the opportunity to conduct this research and providing invaluable guidance throughout my undergraduate degree. His sincerity and understanding guided and encouraged me to continue discovering the unknown. The door to Dr. Huber's office was always open whenever I ran into trouble or needed help. He consistently allowed this paper to be my own work but steered me in the right direction whenever he thought I needed it.

I would also like to acknowledge and thank my committee members, Dr. Koster and Ms. Wone, for their constant help throughout the writing portion of this thesis. Without their help, this thesis would not be readable. I am gratefully indebted to all mentioned for their constant support, this thesis would not have been possible without them. Thank you.

DEDICATION

This thesis is wholeheartedly dedicated to my parents, Chad and Julie, who have been my source of inspiration for all I do. They give me strength and continuously provide me with their moral, spiritual, emotional, and financial support. Without their help, all that I have accomplished and will accomplish would not be possible, and I cannot express my gratitude with words.

To my father: I have always wanted to be exactly like you with a strong drive to put my all into everything I complete. Thank you for teaching me hard work and determination. Also, my college graduation is this year. – Your little buddy

CHAPTER ONE

Introduction

Importance of Understanding Influenza

Influenza is a highly contracted and contagious viral disease, infecting 5-20% of the world population each year (WebMD, 2020). According to the World Health Organization, tens of millions of people are infected with influenza during the flu season. Serious complications are rare for people infected with influenza, however, in the United States, there are more than ten thousand deaths due to influenza infection each year (Center for Disease Control and Prevention, 2020). According to the Center for Disease Control and Prevention (CDC), during the 2019-2020 flu season, starting October 1, 2019, there were an estimated 32 million cases of influenza-like illnesses globallly. In the most recent flu season (2019-2020), there were 310,000 hospitalizations and 18,000 deaths linked to influenza, and the season has not concluded as of the writing date (Center for Disease Control and Prevention, 2020).

In most cases, influenza infections are not fatal. However, in some cases, treatment is needed to prevent further complications. If influenza is allowed to progress, moderate and severe secondary infections may arise. These progressive infections can develop into bronchitis, pneumonia, or sinus infections (Miko et al., 2019). Sinus and ear infections are examples of moderate complications that can arise from influenza. Serious complications that are triggered by an influenza infection include myocarditis, encephalitis, myositis, and multi-organ failure (Center for Disease Control and Prevention, 2019). Not only can influenza infection trigger secondary infections, it may

make chronic medical conditions worse (Center for Disease Control and Prevention, 2019). Since influenza is a respiratory infection, patients with underlying respiratory complications possess a substantial risk when contracting influenza.

There are many preventative procedures that should be considered for influenza, such as increased personal hygiene and receiving an annual flu vaccination. Flu vaccines have become increasingly popular and are easily accessible to the general public. The CDC estimates the flu vaccine as 40 to 60 percent effective among the overall population during seasons when circulating influenza viruses are well-matched with the strains included in the vaccine (Center for Disease Control and Prevention, 2020). In the most recent flu season (2019-2020), the CDC estimates that the vaccine has only been about 45 percent effective. With the prevalence and known history of influenza, one might think that a more accurate and effective vaccination could be accessible, however, since it is difficult to predict which of the multiple strains or subtypes of influenza will be circulating in a given year, constant research is needed to prepare for the upcoming flu season.

History of Influenza A and B in Humans

Influenza first became recognized in 1889 as a bacterial infection, starting as an epidemic in Bukhara, Uzbekistan, that developed into a pandemic of Russian influenza within the same year (Erkoreka, 2009). An epidemic is defined as a disease that affects a large number of people within a community, while a pandemic is defined as an epidemic that has spread over multiple countries or continents (Intermountain Healthcare, 2020). The classification of influenza from an epidemic to a pandemic within the same year demonstrates the high transmissibility of the virus. During this time, railways and steam

trains were increasing in numbers, spreading the disease at alarming rates. A few years after the influenza pandemic had circulated the world, most clinicians accepted that "Pfeiffer's bacillus", a bacterium, was the leading cause of influenza. In 1918, influenza continued to spread around the world, infecting billions of people. During this wave, an estimate of 50 million to 100 million people died as a result of influenza infection (Hageman, 2020). Society was unable to keep this infectious disease under control with the therapies they were using to approach this pandemic. However, in 1933, the National Institute for Medical Research was able to identify influenza as a new class of pathogenic viruses (Brennan, 1987). This new classification of pathogens allows a way to distinguish different viral strains utilizing the haemagglutinin (H) and neuraminidase (N) antigens on the virus coat. In subsequent years, different strains such as the Asian flu (1957, H2N2), Hong Kong flu (1966, H3N2), and the 1976 swine flu (H1N1) demonstrated how new strains of influenza could emerge from livestock and wild animals.

Of the four recognized influenza types, A, B, C, and D, influenza A causes the most morbidity in humans (Seladi-Schulman, 2019). Influenza A viruses (IAVs) are the only known influenza viruses to cause flu pandemics around the world (Center for Disease Control and Prevention, 2019). This strain of influenza can be found in many different species including but not limited to humans, birds, and swine (Center for Disease Control and Prevention, 2018). Due to the large diversity of hosts and influenza's ability to change genetically, influenza A viruses are very diverse. Although influenza A is commonly thought to be one strain, many different subtypes arise to cause global epidemics. These viruses are divided into many subtypes based on the surface proteins H and N. There are 18 different H subtypes and 11 different N subtypes (Seladi-Schulman,

2010). There are currently 131 subtypes detected in nature, with A (H1N1 and H3N2) circulating in humans most predominantly (Center for Disease Control and Prevention, 2019). The current H1N1 virus strain emerged in the spring of 2009 and has continued to undergo small genetic changes that yield changes to its antigenic properties, making this virus hard to target and allowing for elevated infectivity (Cheng & To, 2012). It is estimated that influenza A accounts for 75 percent of confirmed seasonal infections. (Seladi-Schulman, 2019).

While influenza B viruses (IBVs) are unlike influenza A viruses in terms of genetics; symptoms associated with these two viruses are generally similar (Anthony, 2018). IBVs are classified into two lineages: B/Yamagata and B/Victoria (Yang et al., 2012). Given the fact that Influenza B viruses mutate at a much slower rate than IAVs in terms of genetics and antigenic properties, and that the primary host for IBV is human, there is a reduced chance of a possible pandemic concerning influenza B. Influenza B is commonly spread from human to human and is highly contagious similar to influenza A (Center for Disease Control and Prevention, 2019). While influenza A accounts for nearly 75 percent of confirmed flu cases, influenza B accounts for the remaining 25 percent (Seladi-Schulman, 2019). Influenza B is still a strain that raises concern, however, as influenza B viruses have been perceived to circulate in particular time periods and geographical areas. The two strains of influenza B, Vic87 and Yam88, have been known to decline in infection magnitude and then re-emerge later in time, causing epidemics of influenza B viruses to fluctuate both periodically and geographically (Yang et al., 2012). Influenza B may not appear to be as high of a threat as IAV due to the slower evolution and antigenic drift, however, various mechanisms of insertion, deletion, and reassortment

within different lineages increase the genetic diversity of influenza B (Dudas et al., 2014). Nonetheless, both influenzas raise concern to the public and the research behind the two types of influenza is constant and continuous.

Ferrets as a Model System for Influenza Study

For many years prior, mice have been used as a model system to study influenza viruses (Belser et al., 2011). The individual animals were low cost, readily available, and had extensive availability for the use of reagents and for transgenic lines with targeted gene disruptions. It was later found that most human influenza strains do not replicate efficiently in mice without prior virus adaptation, and the clinical signs commonly found in humans were not present in mice (Belser et al., 2011). Mice were deemed a poor model for the study of influenza, thus leading to the guinea pig model. Guinea pigs support the replication of influenza without prior adaptation, but like mice, do not replicate the clinical signs experienced by humans (Belser et al., 2011). Non-human primates were then used as a model, but proved to be exceedingly large and costly, and ethical considerations limited the use these species as a model (Belser et al., 2011). An improved model was needed, and the ferret was then introduced to the laboratory setting.

The use of ferrets for the understanding of influenza viruses has broken ground in scientific research. Ferret models (*Mustela putorius furo*) have been established for many viruses that lead to respiratory infections (Matsuoka et al., 2009). Ferrets are a common mammalian model for certain studies as they are relatively small in size and they possess the ability to emulate numerous clinical features associated with human disease (Matsuoka et al., 2009). This is especially the case in regard to influenza. They provide an ideal model for an influenza study as they are naturally susceptible to human-adapted

influenza viruses, and when infected they display similar symptoms as humans (Matsuoka et al., 2009). These symptoms include fever, lethargy, nasal discharge, sneezing, and coughing. Not only do the two species exhibit similar clinical signs, the lung physiology of humans and ferrets is also relatively similar (Belser et al., 2019). The human and avian influenza viruses show similar patterns of binding to sialic acids on the cell surface, which are distributed throughout the respiratory tract in both humans and ferrets (Besler et al., 2019). Ferrets display a high susceptibility to influenza virus, and in 1933, it was shown that an infected ferret is able to transmit the virus to healthy individuals within the ferret species (Smith et al., 1933). Human and avian viruses replicate efficiently in the respiratory tract of ferrets without prior adaptation, and enhanced pulmonary spread of virus is seen following infection with some highly pathogenic avian influenza (HPAI) viruses (Belser et al., 2011). Numerous symptoms found in humans during a seasonal influenza outbreak were also present in ferrets following experimental inoculation with these strains via the intranasal route (Moore et al., 2014). Elevated body temperatures can be detected as early as one day post-inoculation and nasal discharge along with sneezing can be observed up to seven days post-inoculation (Zitzow et al. 2002). Upon collection of data, the blood and/or tissue samples can be collected from an inoculated ferret, and the presence of virus can be quantified. The use of ferrets in the study of influenza has proven to be highly successful and has offered crucial information for the understanding of influenza.

Using ferrets as models to study the infectivity of influenza A and B in the upper and lower respiratory tract have proven useful to the understanding of these viruses. Studies were previously conducted using autopsy material from humans, so only changes

associated with lethal outcomes and late-stage disease had been characterized (Taubenberger & Morens, 2008). The infection of influenza goes far beyond this, varying with clinical progression and length of the disease before death. Concern about the emergence of an influenza pandemic caused by an HPAI virus (influenza A strain) makes reviewing the pathology of previous pandemics relevant. Both types of influenza display a standard pattern of infection in humans with an exponential increase in virus titer, which peaks around two to three days post infection, followed by an exponential decrease until days six to eight, with the virus titer ultimately undetectable in both the upper and lower respiratory systems (Baccam et al., 2006). Aside from this, influenza A and B are known to differ in the rate of infectivity and the direction in which the virus travels, loosely defined as kinetics. The difference in kinetics between the two types is defined around the region of infection in the respiratory tract, specifically the upper and lower portions.

Unlike influenza B, influenza A replicates in both the upper and lower respiratory tract (Baccam et al., 2006). IAV can be detected in both the nasal passage and in the lower respiratory tract (lungs), for the duration of nine days post-inoculation (Kim et al., 2015). The kinetics of influenza A are commonly studied, but information concerning the kinetics of influenza B is not nearly as abundant. Few studies with reliable data have been conducted concerning the lower and upper respiratory infections of influenza B. In a study by Kim (2015), the presence of virus was found in the nasal turbinate, but all samples collected showed no presence of influenza B in the trachea or lung. This is intriguing as influenza virus is thought to replicate in the epithelial cells throughout the respiratory tree (Taubenberger & Morens, 2008). People with chronic lung disease are

cautioned by the CDC to be cautious during the flu season as they are more at risk for the likelihood of severe disease and death. If influenza B is deemed to be absent in the lungs, those with chronic lung disease that have contracted an IBV can rest assured knowing that the likelihood of mortality is lowered. The absence of knowledge concerning the kinetics of influenza B suggests that more research is needed if the number of people infected by influenza is to decrease.

Influenza and the General Public

With the current coronavirus disease of 2019 (COVID-19) pandemic, it is evident why viral kinetics and the possible outcomes of viral infections should be studied. Every year, seasonal influenza makes a return with the possibility of catastrophic consequences when pandemics occur. While the importance of influenza pandemics has been well-noted since 1918, the virus continues to infect thousands and kill an average of 500,000 people globally each year (Fauci & Collins, 2012). The cases of influenza during a "normal" influenza season in the United States can reach extreme numbers of 20 to 50 million cases of influenza and influenza-like illnesses (Clayville, 2011). The virus is continuously undergoing extensive genetic changes in addition to possessing the ability to transfer between species (Webster & Govorkova, 2015). This concept leads to high vulnerability in humans. If an event similar to the previously described occurs, a global health disaster can arise, such as the 1918 influenza pandemic. The 1918 pandemic killed an overwhelming fifty to one hundred million people worldwide (Institute of Medicine, 2005). Not only did an extreme mortality rate affect the public, the world encountered enormous social and economic disruption. Influenza circulates widely and is constantly

evolving toward pandemic capability, as was seen in the pandemic of 1957, 1968, and 2009. This indicates a clear danger of future pandemics caused by the influenza virus.

One of the goals present in pandemic influenza research is to recognize and predict how viruses are evolving in wild animals toward a phenotype that is threatening to humans (Fauci & Collins, 2012). This research could allow society to stay one step ahead of the virus and be able to prepare for any future outbreaks. However, due to the constant evolution of the virus, continuous research is needed to keep up with the genetic changes undergone by influenza. If staying one step ahead fails and an outbreak occurs, information on how to decrease mortality of the virus in those already infected is crucial as well. Influenza in its entirety must be studied to prepare for a large outbreak, this includes thorough study of the kinetics of influenza infection. Currently, little is known about the kinetics of influenza during infection due to its constant genetic evolution. Numerous studies are in place to try to comprehend the entirety of influenza and by the analysis and discovery of new information, influenza infection can be hindered or altogether stopped through the development of new antiviral therapies.

Purpose

Many studies have been conducted on the infectivity and kinetics of influenza A, however, influenza B has not been studied as thoroughly. It has been determined that influenza A has the ability to first infect the upper respiratory tract while later traveling to the lower respiratory system. Influenza B has been determined to infect the upper respiratory system, however less is known about its activity in the lungs. The kinetics of influenza A were tested and verified through this research and the kinetics of influenza B were investigated. The kinetics of influenza B were discovered and analyzed through the

use of ferrets and numerous laboratory procedures. Identifying and understanding the kinetics of influenza B infections will provide crucial information toward the understanding of the virus and could ultimately lead to development of more effective antiviral therapies and vaccines.

CHAPTER TWO

Materials and Methods

Timeline of Events

Male ferrets from an influenza free ferret colony at Marshall Farms were brought to the University of South Dakota (USD), Sanford School of Medicine's animal facility. Ferrets were acclimated for fourteen days, then tested to confirm that the antibody levels were in compliance with titer levels against the three viruses used in this study. All of the procedures and experiments were conducted within biosafety level 2 facilities and in accordance with parameters set individually by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of USD. The experiment took place over a course of nineteen days post-inoculation. Days -4 to Day 0 were spent preparing, replicating, and plating cells for the remainder of the study. On day 0, different groups of ferrets (n=4) were inoculated with *B/Phuket/3073/2013 (BYam), A/California/4/2009-H1N,* and *B/Brisbane/60/2008 (BVic)*. On days one through ten, nasal washes were collected from the four ferrets and Madin-Darby Canine Kidney (MDCK) cells were inoculated with nasal washes to determine whether virus was present in the nasal area. To determine presence of virus, the MDCK cells were incubated for a period of 72 hours before the virus titer was read and quantified. To confirm the presence of virus, individual wells of the diluted nasal wash samples (n=4 per dilution) were tested for their ability to bind red blood cells, indicating presence of virus in the well. Upon completion of all data collection, the results were then analyzed and compared.

MDCK Growth Medium

MDCK growth medium was essential to support cellular growth in an artificial environment, providing nutrients necessary for cultured cells to survive and proliferate. Growth medium also provides the correct osmolality and pH for cells to thrive and replicate. Serum-containing, minimum essential medium (MEM) was used for this study. MEM includes higher concentrations of amino acids allowing for a closer approximation to the protein composition of mammalian cells. Growth medium was used for the growth of healthy cells as well as an additive to ensure MDCK cells reached a confluency of $3x10^5$ MDCK cells per 500 µl of media. This allowed the cells used for infection with nasal wash to remain at similar quantities for each sample dilution analyzed. All cell culture techniques were performed under a sterile hood using aseptic techniques to ensure the solution was free of all microorganisms by sterilization. A 500 mL sterile filter-top (Corning 430626) was attached to a one-liter sterile bottle, later connecting the two components to a pump system. To the filter-top, 100 mL 10X MEM (GIBCO 11430), 50 mL of Fetal Bovine Serum (FBS)- Hyclone (previously heated-inactivated at 56°C for 30 minutes), 10 mL of Antibiotic/antimycotic solution and 10 mL MEM Vitamin solution was added to the filter-top. Fetal Bovine Serum was included because of its high quantity of hormones, carrier proteins, and macromolecular proteins allowing for growth enhancement of the MDCK cells. Antibiotic/antimycotic was added as a prophylactic to prevent contamination, and vitamins were added to the growth medium due to the inability of MDCK cells to synthesize vitamins independently. Ten mL L-glutamine was added to serve as an auxiliary energy source, providing nitrogen for the synthesis of proteins as the cells are rapidly dividing. One mL Gentamicin (10 mg/cc) was added for

its anti-bacterial efficacy. The total volume of the sterile filter-top was brought to 500 mL with ddH_2O (approximately 331 mL). Once filtration was complete, the pump was stopped and the resulting pressure was released. To the empty filter-top, 30 mL Sodium Bicarbonate ($NaHCO_3$) was added to maintain the pH in the presence of 4-10% carbon dioxide. The volume was brought up to 500 mL using approximately 470 mL ddH_2O (double-distilled). Upon completion of this procedure, the color of the MDCK Growth Medium was analyzed for a correct pH balance of 7.4. The contents of the bottle were then stored at 4°C.

MDCK Infection Medium

Along with MDCK growth medium, MDCK infection medium is also essential. The infection medium acts as a viral infection enhancer that allows the facilitation of viral penetration, ultimately enhancing productive viral infection of MDCK cells. This procedure was performed under a sterile hood using aseptic techniques. The filtration system was assembled, in the same way as for the Growth Medium. To the sterile filter-top, 100 mL 10X MEM, 40 mL 7.5% Bovine Serum Albumin (BSA)-Sigma (15 g BSA in 200 mL ddH_2O), 10 mL Antibiotic/antimycotic solution, ten mL MEM Vitamin Solution, ten mL L-glutamine, and 1 mL Gentamicin (10mg/cc) was added. The volume was brought to 500 mL with 329 mL ddH_2O. Thirty mL Sodium Bicarbonate was added to the filter-top along with 470 mL ddH_2O, the pump system was started and upon completion of filtration, the medium was mixed and analyzed for correct color, indicating pH. The contents were stored at 4°C.

Cell Culture

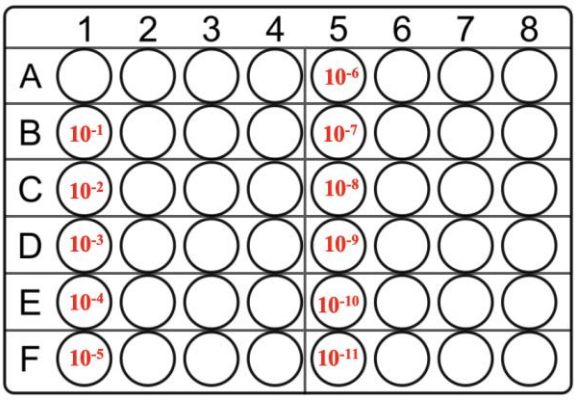
Cells were plated using 48-well sterilized plates. The flask containing MDCK cells and growth medium was viewed under a microscope to ensure a monolayer was produced during cell growth and replication. The growth medium was removed from the cell flask and the cells were washed once with sterile PBS. Washing with PBS allows for removal of dead cells and excess serum in the growth medium that inhibits trypsin. The cells were harvested using 9 mL sterile PBS and 1 mL 0.5% trypsin, adding this solution to the flask, and allowing incubation for 1 hour. Trypsin allows for the removal of calcium from the cell solution, breaking and separating the cadherins in which the cells are held together as well as cutting focal adhesions that anchor the cell to the culture flask (Brown et al., 2012). After incubation, the cells were visualized to ensure the cells were suspended in solution. An aliquot of trypsinized cells was collected and centrifuged for three minutes at 2500 rpm. The supernatant was removed completely so no residual PBS+trypsin covered the cell pellet. The pellet was resuspended in 10 mL of MDCK growth medium. In a small test tube, 400 µL of MDCK growth medium was added with 100 µL of the resuspended cell solution. To ensure confluency of live cells and to reach the desired cell count, 500 µL of Trypan Blue was added to the solution. Trypan blue is a dye used to quantify live cells by labeling dead cells exclusively. Because live cells have an intact cell membrane, trypan blue cannot penetrate the cell membrane of live cells and enter the cytoplasm. In a dead cell, trypan blue passes through the porous cell membrane and enters the cytoplasm. Ten µL of this solution was added to a hemocytometer and was visualized under a microscope. The intact cells were counted and MDCK growth medium was added to achieve the desired density of cells per well, 3×10^5. The MDCK cells were

then plated on a 48 well plate, with 500 µL in each well. Due to the high growth rate of the cells, the cells were split every three days to allow for appropriate space for growth.

$TCID_{50}$

One method used to verify the viral titer was the fifty-percent tissue culture infective dose ($TCID_{50}$). This procedure signifies the concentration at which 50% of the cells are infected. To begin the $TCID_{50}$, the collected nasal wash was diluted using 10-fold serial dilutions. To obtain the desired dilutions, 900 µL of MDCK infection medium was added to a series of ten disposable glass test tubes. After adding medium, 100 µL of the desired virus sample (nasal wash or lung) was added to the first test tube, to obtain a total volume of 1 mL. The samples and all test tube dilutions were kept in an ice bath during the procedure to ensure the infectivity was kept intact. The samples were vortexed before addition to the plate to ensure proper creation of a homogeneous mixture. From the MDCK cells plated a day prior, the medium of the MDCK monolayers was removed with a glass pipet. The cells were washed with two separate additions of sterile 1X PBS (pH=7.43). After the second wash and removal of PBS, 50 µL of the dilutions were added to their respective wells containing the confluent MDCK monolayers. Nasal wash dilutions were added starting with medium as a control and then proceeding to add the lowest dilution first. Adding the lowest dilution first ensured a higher concentration of infected nasal wash was not carried to those with lower concentrations. Medium was used as a control in wells A1-A4, as displayed below in *Figure 2.1*.

Figure 2.1: 48-well plate configuration for serial dilutions of virus stock used for $TCID_{50}$ protocol. Red values indicate dilution of nasal wash.



The cell plates were then incubated at either 37°C (for IAV) or 33°C (IBV) for one hour, allowing for optimal infectivity, with gentle tapping every 15 minutes to ensure the sample was evenly distributed across the monolayer of cells. Using 40 mL of MDCK infection medium, 40 µL of TPCK-trypsin was added to the test tube. The working solution was made for a ratio of 1 µL of 1 mg/mL stock solution per milliliter of MDCK infection medium. The cell plates were removed from the incubator and the virus inoculum was aspirated off the MDCK monolayer, from most dilute to least, to prevent the dragging of higher concentrated virus across the samples. After aspiration, 500 µL of contents containing MDCK infection medium supplemented with TPCK-trypsin, was added to each individual well. The plates were incubated at 37°C (IAV) or 33°C (IBV) for 72 hours and were observed daily for cytopathic effect (CPE).

Preparation of CRBCs

Influenza viruses have an envelope protein, known as hemagglutinin, that binds to sialic acid receptors on cells. The virus will also bind to red blood cells causing the formation of a lattice. The binding to red blood cells is the basis of the rapid assay that

determines the quantity of virus present. To prepare the red blood cells needed for the HA Titer, chicken red blood cells which are shipped in Alsevers solution to prevent coagulation of the blood cells, were resuspended and transferred in 12 mL increments into two 15 mL conical tubes. The cells were centrifuged using an Eppendorf Centrifuge 5910 R, for four minutes at 1,338 rpm (357xg) and were kept at 25°C. After centrifugation, the supernatant was removed, taking special care to preserve the RBC layer, but removing the white blood cell (WBC) layer. Ten mL of (1X) PBS was added to each tube with the cell layer. The mixture was then centrifuged again for four minutes at 1,338 rpm (357xg) at 25°C. The supernatant was then disposed carefully to preserve the cell layer, but removing all PBS added previously. Ten milliliters of (1X) PBS was added to each of the tubes. The mixture was centrifuged for 20 minutes at 800 rpm and kept at a temperature of 25°C. The supernatant was removed once again, to ensure no PBS remained in the conical tube. In a sterile bottle, 100 mL of sterile (1X) PBS was added and 0.5 mL of RBC from the conical tube was added to the PBS. The RBCs were carefully resuspended using a pipette, until the mixture was homogenous. Upon completion, the 0.5% CRBCs were stored at 4°C.

HA Titer

This test relies on the hemagglutination activity of influenza hemagglutinin and the ability of HA-specific to agglutinate erythrocytes. Influenza virus particles contain a homotrimeric glycoprotein on their surface, hemagglutinin (HA), which causes erythrocytes to agglutinate. Hemagglutinin binds to sialic acid receptors on the cells and will also bind to erythrocytes causing the formation of a lattice. This property allows for the quantification of virus present in a sample.

After the 72 hour incubation, the MDCK plates previously inoculated with nasal wash were transferred to a 96 well plate. A round-bottom 96-well plate was used to allow the red blood cells to settle in the bottom of the well and to decrease the surface area and volume of individual wells. From the $TCID_{50}$ plates, 50 µL from each incubated well was transferred to a new well in the 96-well round-bottomed plate. Following the transfer, 50 µL of 0.5% CRBC solution was added to each well to bring the volume to a total of 100 µL. The plates were allowed to incubate at room temperature for 30 minutes. The plates were then observed and analyzed, with settling of the erythrocytes into a pellet indicating the absence of a virus (negative). If settling was not observed and agglutination occurred, this indicated that the virus was able to bind to the CRBC solution, indicating that virus was present in the specific sample at the indicated dilution.

CHAPTER THREE

Results and Data

Clinical Data

Weight, temperature, and infection of the upper and lower respiratory system were monitored daily in the experimentally infected ferrets. According to a study conducted by Lee (2010), the most frequent clinical symptoms of influenza infection included fever, nausea, and diarrhea. Nausea and diarrhea would lead to a decrease in weight, an easy variable to measure in a laboratory setting. Increased body temperature, pyrexia, was proven to be the most frequent clinical symptom noted by those infected with influenza (Plaza, 2016). Finally, $TCID_{50}$ and HA titers are ways to both confirm and quantify the presence of influenza within the respiratory system. Together, these variables were able to characterize the ferret response and virus shedding and localization after infection by two types of influenza. The strain of influenza A used within this study includes A/California/4/2009-H1N1 (Cal09). The two strains of influenza utilized in this study consist of B/Phuket/3073/2013 (BYam lineage, B/Phuket) and B/Brisbane/60/2009 (BVic lineage, B/Brisbane).

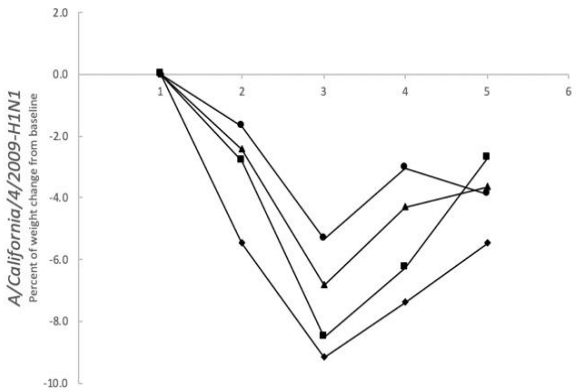
A/California/4/2009-H1N1

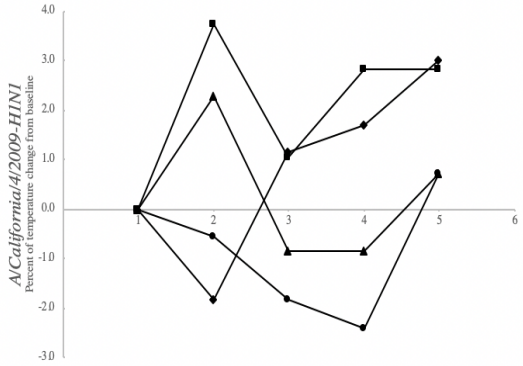
The weights were recorded for ferrets infected with the Cal09 virus. This influenza A virus is similar to what was used in previously published studies and provides a basis for the research conducted on influenza B. The animals were monitored for weight loss daily, in grams, over a period of nine days. The baseline used for the comparison of data is defined

as the data collected on the day of inoculation, representing the norm. All of the ferrets inoculated with the IAV showed a decrease in weight, with peak percent weight losses being 9.19% and 8.52%. This data is presented in *Figure 3.1* below. A value of (1) on the x-axis indicates day zero, in which the ferrets were inoculated. All ferrets included in the experiment concerning infection with influenza A exhibited a decrease in weight starting the day after inoculation and remained below the baseline for the duration of the study. Although the weights remained below the initial weight prior to inoculation for the entirety of the experiment, 43.75% of all weights noted, exhibited weight gain when compared to the weight recorded on the previous day. When compared to the baseline weight recorded, the ferrets exhibited weight changes of -2.72%, -5.50%, -3.91%, and -3.67% on the day concluding this experiment. Rectal temperatures were also recorded over the course of the experiment. A normal temperature for a ferret ranges between 37.8 to 39 degrees Celsius (Leesville Animal Hospital, 2016). The data collected for temperature comparison throughout the experiment can be seen below in *Figure 3.2*. The animals exhibited body temperature changes ranging from 1.39 degrees Celsius to -0.95 degrees Celsius from the initial temperature recorded on day 0. Of the sixteen temperatures recorded post-inoculation, 62.5% of all temperatures recorded, showed an increase from the baseline through the duration of the study. Although two ferrets exhibited decreases in body temperature for day one post-inoculation, the temperatures eventually rose to above normal body temperature recorded prior to infection. All ferrets inoculated with Cal09 showed an elevated body temperature at the end of the experiment, day four (depicted by five on the x-axis in *Figure 3.2*).

***Figure 3.1 (left)**: Percent of weight change in ferrets infected with Cal-09 virus.*

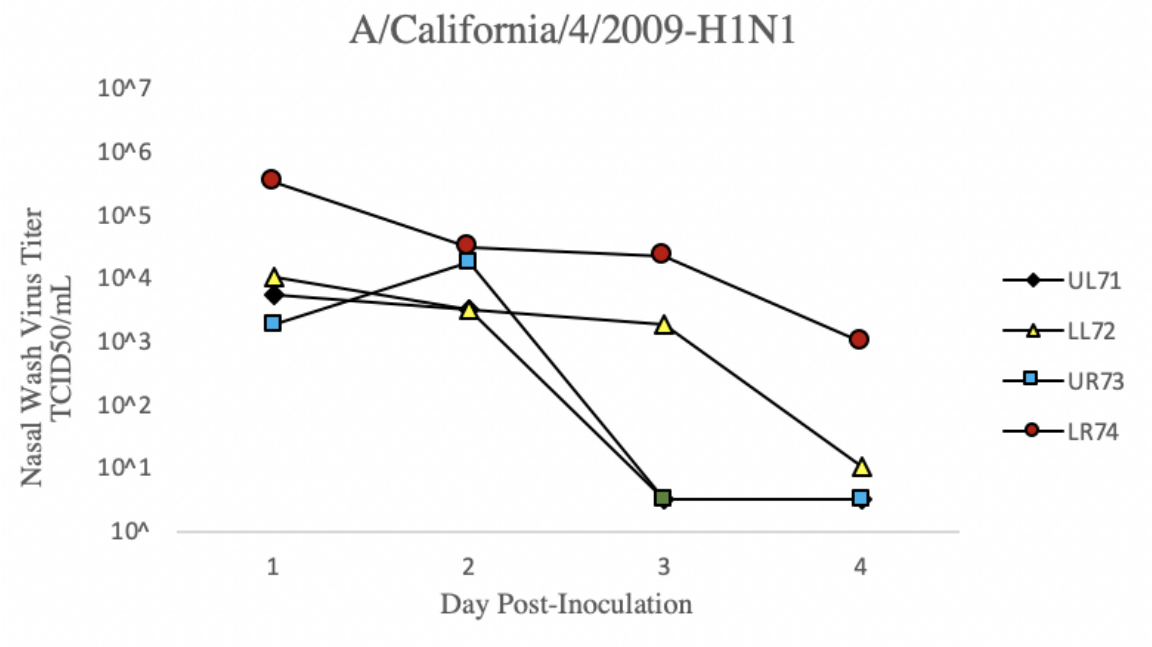
***Figure 3.2 (right)**: Percent of temperature change in ferrets infected with Cal-09 virus.*





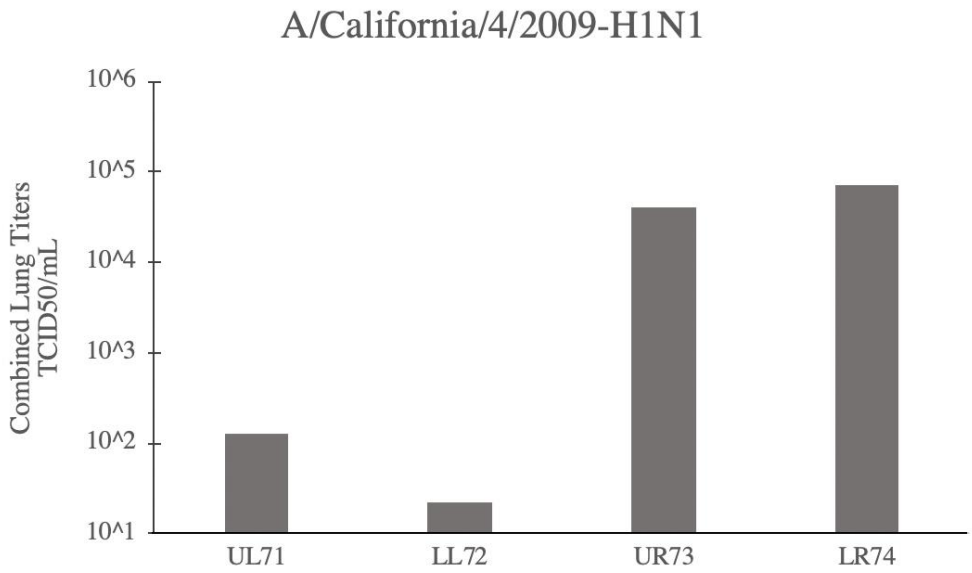
In addition to weight and temperature change, a virus titer was conducted for the nasal wash and the lungs. The data showed an initially large presence of influenza virus in the nasal wash. The $TCID_{50}$ values for the ferrets infected with Cal09 can be compared and viewed below in *Figure 3.3*. The nasal wash data from the four ferrets were compared based on the $TCID_{50}$ value of the virus. On day one, the four ferrets showed an average $TCID_{50}$ value of $10^{4.5}$ $TCID_{50}$/mL. This value denotes the peak concentration of Cal09 taking place 24 hours after inoculation. The average $TCID_{50}$ value then proceeded to steadily decrease over the course of four days to a $TCID_{50}$ value of near 10^{1} $TCID_{50}$/mL, indicating little to no viral activity in the nasal wash near the end of the experiment.

***Figure 3.3**: TCID$_{50}$ values for ferrets UL-71, LL-72, UR-73, LR-74, over the course of four days.*



Along with the nasal wash titer, data were collected to observe the presence of Cal09 in the lungs of the four ferrets. Lung titers were conducted upon euthanasia of the animals, taking place after nasal wash data was completed. Different sections of the lung were collected and combined into a single homogenate. The data in *Figure 4.3* shown below, illustrates the TCID$_{50}$ combined lung titer for ferrets inoculated with Cal09. Aside from the values obtained, these data show that the virus was able to reach not only the nasal wash, but also the lungs. Virus in the nasal turbinates decreased over the course of four days post-inoculation, however, virus titers performed on the lung samples after nasal wash data collection showed substantial viral activity, indicating the movement of influenza A from the upper to the lower respiratory tract. Upon lung data collection on day four, the data showed an average TCID$_{50}$ value of $3.7x10^4$ TCID$_{50}$/mL for the four ferrets, indicating viral activity in the lower respiratory system while virus was no longer detected in the nasal wash.

***Figure 3.4**: Combined lung titers for ferrets inoculated with Cal-09 virus.*



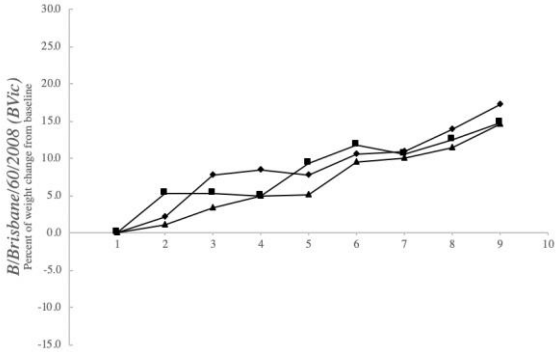
B/Brisbane/60/2008 (BVic)

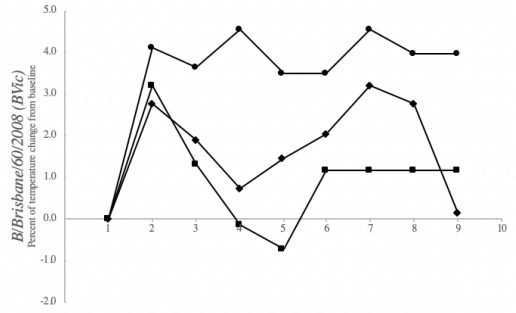
The ferrets infected with *B/Brisbane/60/2008 (BVic)* were weighed on the day of inoculation, and each day for the following eight days. The weights showed an increase from day one to days eight, with the weight remaining above the baseline for all three ferrets throughout the duration of the experiment. These data are displayed below in *Figure 3.5*. When the weights were compared on a day to day basis, 83.33% of the total recorded weights showed an increase from the previous day. The peak weight loss occurred at day seven, with a single ferret exhibiting a weight change of -9.9 grams in 24 hours, but upon conclusion of this experiment, this particular ferret showed a final percent gain of 14.77%, a gain of 133.40 grams. The final weight change from the baseline for the three ferrets upon conclusion of the experiment were 14.77%, 17.19%, and 14.57%. The ferrets were monitored daily over a nine-day period for temperature changes as well. Animals in the B/Brisbane inoculation group exhibited changes in body

temperature that ranged from 1.66 to -0.29 degrees Celsius from the initial temperature taken on inoculation day, shown below in *Figure 3.6*. Of the 24 temperatures recorded post-inoculation, 91.97% percent showed an increase in temperature from the initial value observed on day 0. The elevations were first observed on day one post-inoculation (day two indicated by *Figure 3.6*) and were maintained through day nine in all three animals. The final temperatures taken on day nine for the three ferrets showed a positive value in relation to the baseline.

Figure 3.5 (left): *Percent of weight change in ferrets infected with B/Brisbane virus.*

Figure 3.6 (right)*: Percent of temperature change in ferrets infected with B/Brisbane virus.*

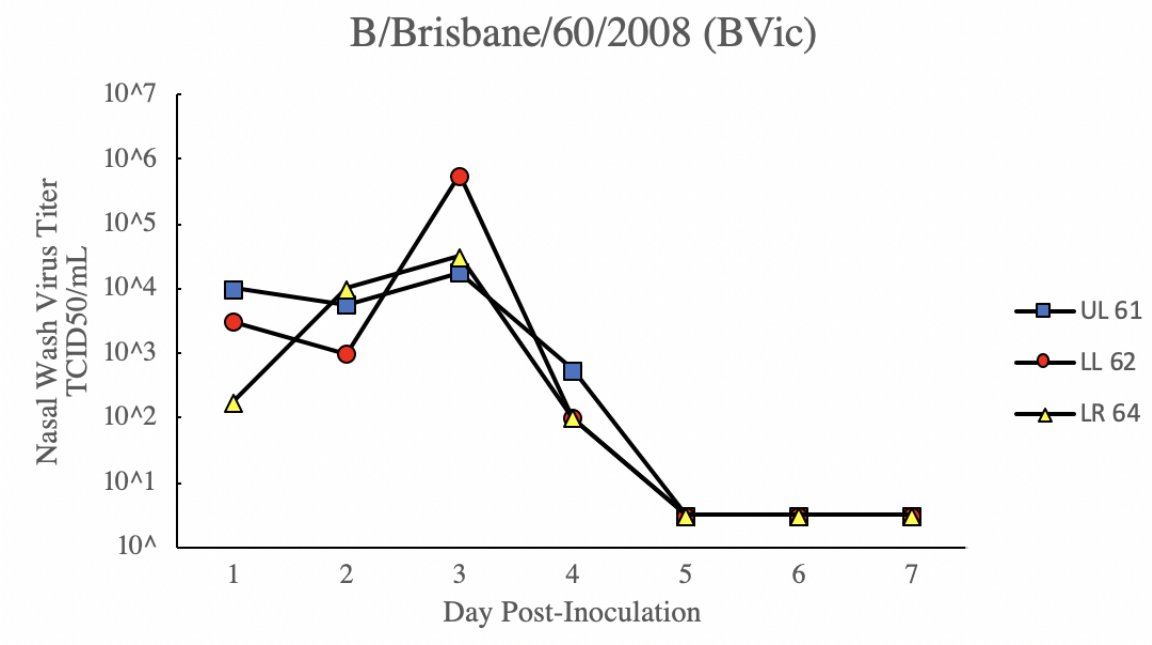




The nasal wash $TCID_{50}$ values were compared for three ferrets over the course of seven days, as shown below in *Figure 3.7.* On day one, the value showed an average of 10^3 $TCID_{50}$/mL for the nasal wash virus titer. The average value then increased until day three, to a value of 10^5 $TCID_{50}$/mL. After day three, the value drastically decreased over the course of two days until a combined average $TCID_{50}$ value of $10^{.5}$ $TCID_{50}$/mL was obtained. The titer value remained at this value for the next three days indicating no further viral activity in the nasal wash. These results indicated that the IBV was present in the upper respiratory system until day five post-inoculation. These results resembled the

results obtained from the Cal09 inoculum group, indicating similar upper respiratory kinetics between the two influenza types. The kinetics between the two types of influenza differed in viral infection time and length of infection. Influenza A appeared to peak in the nasal wash 24 hours after inoculation, but the virus was no longer active in the upper respiratory system at day four. Influenza B, B/Phuket, took three days to reach peak upper respiratory infection, and lasted five days post-inoculation.

***Figure 3.7**: TCID$_{50}$ values for ferrets UL-61, LL-62, and LR-62, over the course of seven days.*



B/Phuket/3073/2013 (BYam)

B/Phuket/3073/2013 (BYam), was the second influenza B strain utilized for this particular study. The B/Phuket virus in the Yamagata lineage, represent one of two lineages of influenza virus (BYam and BVic) that have circulated in humans since the mid-1980's and were included in the 2014-15 seasonal influenza vaccination for the Northern Hemisphere. Cal09 was used to verify the methods and applied as a control group before proceeding with IBV infection. B/Brisbane was used to evaluate the kinetics

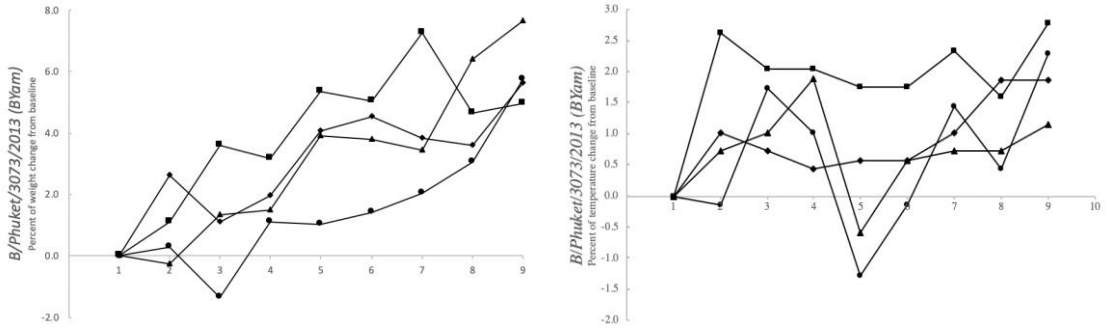
of upper respiratory infection concerning IBV. Similar methods were used for the evaluation of the kinetics of the B/Phuket virus, however a lung titer was included in this experiment to observe viral infection of influenza B in the lower respiratory system. The four ferrets were monitored for nine days, with weight, temperature, nasal wash titer, and lung titer data recorded.

The weight of four ferrets was recorded for nine days post-inoculation. Like the ferrets in the B/Brisbane inoculation group, the weights at day nine were measured as above the baseline, as shown below in *Figure 3.8*. Similarly, a majority of the weights remained above the baseline for the duration of the study, fluctuating during the nine day experiment. The largest decline in weight could be seen on day two, with two ferrets exhibiting an average 1.56% decline in body weight during the 24-hour period. The peak weight change below the baseline exhibited a 1.35% decrease but increased to above the baseline on the day following. Throughout the experiment, 93.75% of the 32 weights recorded, remained at a positive value in relation to the baseline. When compared day to day, 68.75% of the total weights recorded, exhibited an increase. These data indicate a large fluctuation in weight throughout the experiment. Upon completion of the experiment, the final percent weight change for the four ferrets were 6.09%, 8.37%, 6.01%, and 7.37%. Temperature was monitored and recorded for the B/Phuket inoculum group for nine days, shown below in *Figure 3.9*. The temperature changes ranged from 1.06 to -0.45 degrees Celsius from the baseline. The increase in temperature can be seen on the day following inoculation, similar to the B/Brisbane inoculum group. On day five, two ferrets exhibited temperatures below the baseline. The temperatures recorded below the baseline were a minor -1.29%, drastically increasing to at or above the baseline on

day six. The temperatures then remained elevated after day six for the remainder of the experiment. By day nine, all animals held temperatures above the initial baseline. Of the 32 temperatures recorded through the duration of this experiment, 87.5% exhibited a positive value above the baseline.

Figure 3.8 (left)*: Percent of weight change in ferrets infected with B/Phuket.*

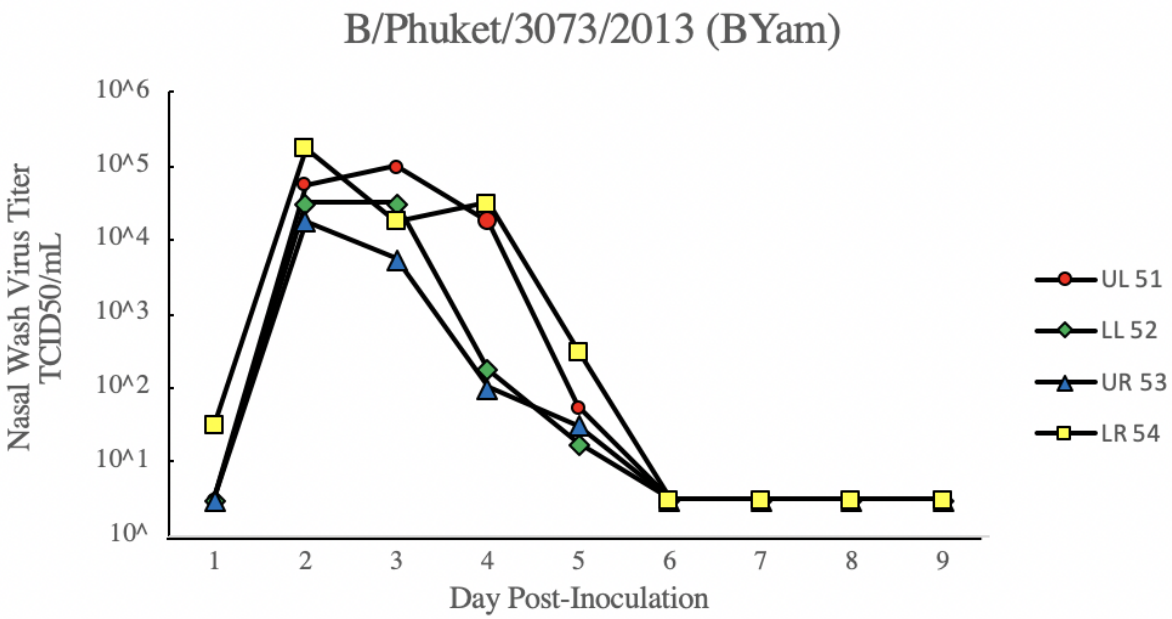
Figure 3.9 (right)*: Percent of temperature change in ferrets infected with B/Phuket.*



Two different B/Phuket inoculum groups were used to examine the kinetics of influenza B. The ferrets 51-54 in the first group were used to collect data regarding clinical symptoms and nasal wash infectivity. Ferrets 81-84 were used to collect data on nasal wash and lung infection. The four ferrets in group one inoculated with B/Phuket showed a low nasal wash $TCID_{50}$ value on day one, an average of 10^1 $TCID_{50}$/mL. The data, shown below in *Figure 3.10* shows a drastic increase until day two post-inoculation, with a value of 10^4 $TCID_{50}$/mL. These data are similar to what was seen in the experiment with B/Brisbane infection. The nasal wash titers then decrease over the course of four days to achieve a value equivalent to day one. The value remains at 10^1 $TCID_{50}$/mL for four consecutive days. This indicates no further B/Phuket activity in the upper respiratory system, similar to what B/Brisbane and Cal09 exhibited. The length of

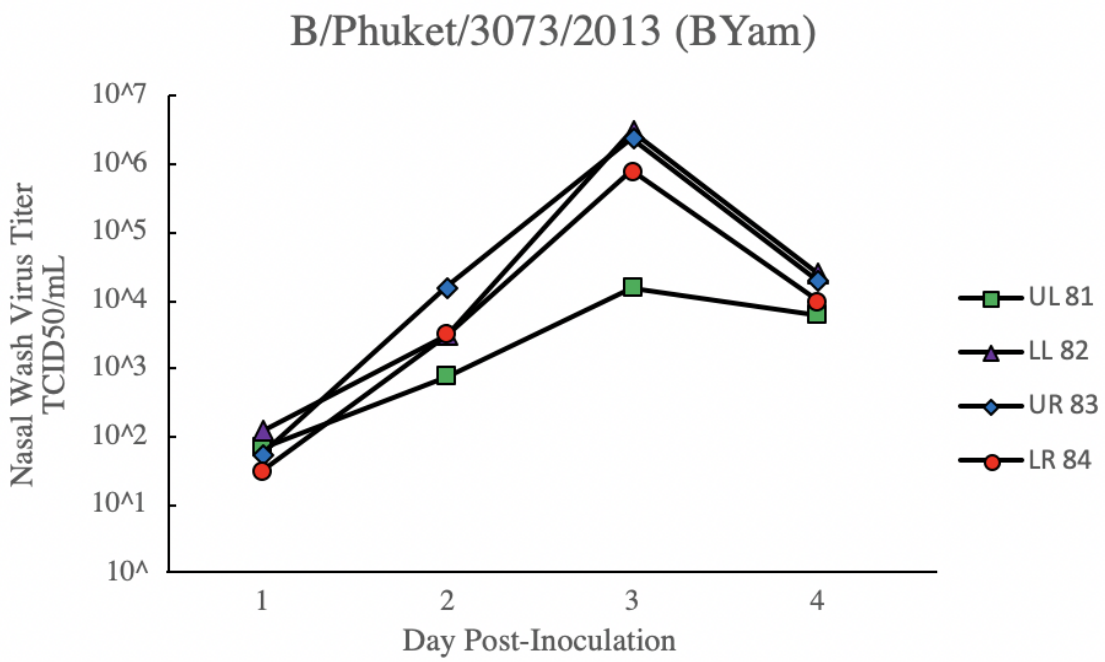
infection is similar to data observed in the B/Brisbane inoculum group, taking course over six days post inoculation.

***Figure 3.10**: TCID$_{50}$ values for ferrets UL-51, LL-52, UR-53, and LR-54, over the course of nine days.*



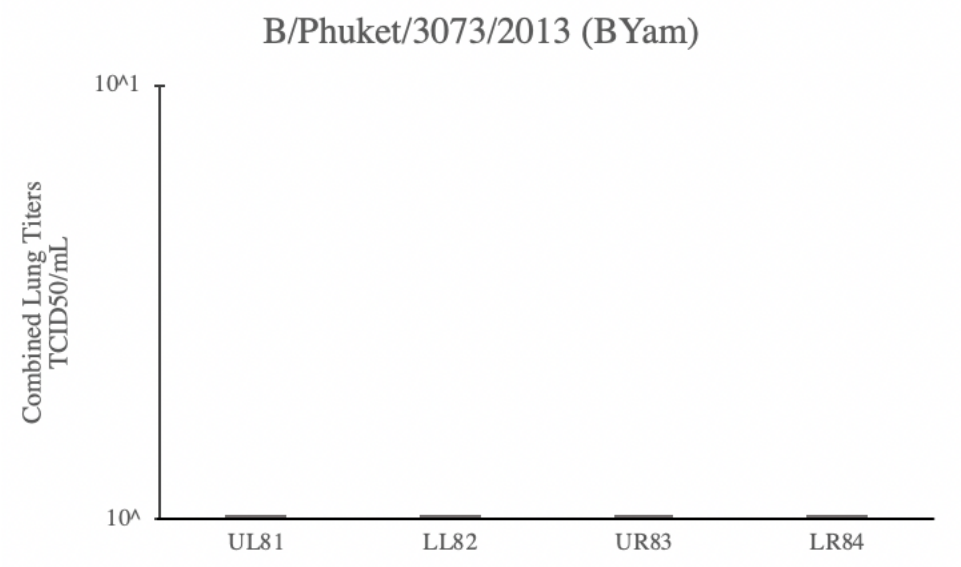
To verify the data collected on B/Phuket, the nasal wash TCID$_{50}$ values for ferrets 81-84, were analyzed and recorded in *Figure 3.11* below. The day one data appeared to be similar to the previous ferrets with a value of 10^1 TCID$_{50}$/mL. The data then, similarly, increased drastically after day one, except now over the course of two days, $1.25x10^5$. This indicated that the virus was able to replicate efficiently and to high titer values. The data then decreased at about the same rate as it increased and a value of $7.5x10^4$ TCID$_{50}$/mL was observed on day four. This confirms the progression and kinetics of B/Phuket in the upper respiratory system. To keep parameters similar to the experiment concerning Cal09, lung samples were collected on day five, not allowing the virus to reach inactivity in the nasal wash.

***Figure 3.11**: TCID$_{50}$ values for ferrets UL-81, LL-82, UR-83, and LR-84, over the course of five days.*



To evaluate the kinetics of an IBV in the lower respiratory system, lung titers were sampled from the second B/Phuket inoculum group. Samples from the B/Phuket group, ferrets 81-84, were harvested on day five post-inoculation. The samples were homogenized, and the tissue homogenates were clarified by centrifugation and titrated in 48-well and 96-well tissue culture plates continag a monolayer of MDCK cells. The data was obtained in the same way used for nasal wash samples. The data obtained, shown below in *Figure 3.7*, show no presence of virus residing in the lungs at day five in the experiment. When compared to Cal09, the lung data provides a difference between influenza A and influenza B in the lower respiratory system.

***Figure 3.12**: TCID$_{50}$ value shows no virus detected in the lungs of ferrets 81-84.*



CHAPTER FOUR

Discussion and Conclusion

Viral Titers

In this study, the kinetics of influenza A and B were observed. The variables used were able to characterize the ferret response and virus shedding and localization after infection with influenza A and B. The influenza A virus used, Cal09, showed viral localization in the upper respiratory tract during the beginning of infection. The viral titer showed a high presence of virus in the nasal wash shortly after inoculation, with the highest virus titer recorded on day one post-inoculation. The viral presence slowly started to decrease over the course of infection, until little trace of virus was detected on day four. Lung homogenates were collected after viral nasal wash activity was observed, showing a positive viral titer for the lower respiratory system. The influenza B viruses, B/Brisbane and B/Phuket, showed different viral shedding and localization. In the nasal wash, the virus titer did not peak until days two or three post-inoculation. The viral titer then decreased over the next few days, with the viral titers indicating viral activity until days five to six. These results express the rate of viral infection in the upper respiratory system, with influenza A infecting at an increased rate, and only lasting around four days. Influenza B, however, took 24-48 hours to reach peak infection, and viral infection lasted approximately five to six days. Viral infection was then noted in the lower respiratory system, with influenza A showing infection in the lungs, and influenza B (B/Phuket) showing no infection in the lungs. These data indicate that influenza A has an increased viral infection and localization rate when compared to influenza B.

Weight and Temperature

The weights observed during influenza A infection appear to corelate with the rate of infection. On the first few days when maximum virus load was indicated, the weights decreased drastically, When virus titers for the nasal wash started to drastically decrease around days two and three, the weights began to increase to near the initial weight prior to inoculation, but still showed an average percent weight change of -3.95%. The data collected indicates that influenza A viral infection does affect weight, but not over a substantial amount of time. In influenza B infection, the weights displayed different characteristics, showing almost opposite results. The weights did not appear to correlate with the degree of viral infection. Instead, increased weights were observed throughout the nine days, regardless of the virus titer for the nasal wash. On day nine, ferrets inoculated with B/Brisbane showed an average percent weight change of 15.51%, with 83.33% of the data showing an increase on a daily basis. This indicates a steady, almost linear increase in weight throughout the duration of the experiment. In ferrets inoculated with B/Phuket, the weights increased similarly. On day nine, the average percent weight gain was 6.96%, with 68.75% of the data displaying an increase in weight on a daily basis. When the influenza B infections are grouped together, it can be concluded that weight gain is observed during influenza B infection.

Aside from different characteristics recognized for weight, temperature for influenza A and B showed similar characteristics. On day one post-inoculation, half of the ferrets infected with influenza A showed a drastic increase in temperature, while the other half showed a decrease in temperature. Similar to this finding, the statistics were the same when temperature was compared on a day to day basis. Throughout the study, 50%

of the data showed an increase in daily temperature while the other 50% showed a decrease in daily temperature. Apart from these fluctuations in temperature observed during the duration of this study, the temperatures on day four after influenza A infection showed an average increase in percent temperature change of 1.81%. The temperature did not show a correlation to viral infection on a day to day basis, but upon conclusion of experimentation, an increased percent change in temperature of all four ferrets indicates a relationship to influenza A infection. The temperature change in influenza B infection was more prominent. In B/Brisbane infection, the temperature appeared increase almost immediately after inoculation and remained elevated throughout the experiment. On day nine, an average percent temperature change of 1.76% was noted. In B/Phuket infection, temperature change was similar. The temperature appeared to increase immediately after inoculation and remained elevated. On day nine, an average percent temperature change of 2.03% was observed. In influenza B infection, it can be concluded that temperature will increase during upper respiratory infection, and will remain elevated for days following, regardless of detection of virus in the nasal wash. In influenza A infection, it can be concluded that temperature may not indicate viral infection on a daily basis, but over time a positive percent temperature change can be noticed.

Why Influenza B?

In a study conducted by Lakdawala et al. (2015), on the kinetics of H1N1 pdm virus A/California/07/2009, the data published resemble the results and methods presented in this study. In the study by Lakdawala, titers were collected and analyzed for days one, three, and five in different parts of the respiratory tract. The Cal09 virus

showed a positive mean virus titer in the soft palate, nasal wash, right mid lung, and the left cranial lung. This indicated that the IAV was able to infect both the upper and lower respiratory systems, similar to what was observed in the data concerning Cal09 presented in this study. In a different study conducted by Moore et al. (2014), the inoculum volume and its effect on clinical disease was evaluated using two strains of IAV. The effects of IAV infection were analyzed using recorded data for weight change, temperature change, nasal symptoms, and infection in the upper and lower respiratory system. The samples quantified using $TCID_{50}$ values displayed, over a course of six days post-inoculation, that the weights of twelve ferrets appeared to decrease at a relatively steady rate (Moore et al., 2014). This data presented by Moore confirms the results obtained in this study. In Moore's study, the temperature appeared to differ drastically from the baseline over the course of six days post-inoculation, in most cases, remaining above the baseline over the duration of data collection (Moore et al., 2014). The data presented in this current study, resembles the data Moore published as well. The $TCID_{50}$ values for this current study read the highest in the nasal turbinates, but also showed a positive value in the combined lung indicating infection in the upper and lower respiratory tract. The same ideas and methods from Moore and Lakdawala were applied to this current study, however, for this particular study, IBV infection was included. The studies from Lakdawala and Moore provide a basis for the study of the kinetics concerning influenza A and lead to the study of kinetics involving influenza B infection.

Discussion

Influenza is a communicable disease that affects both the upper and lower respiratory tract. There are four subtypes of influenza, some of these infecting humans and others specific to different species (Center for Disease Control and Prevention, 2019). Influenza virus is transmissible through respiratory droplets expelled from the mouth and respiratory system (Boktor, 2019). Influenza can be transmitted before the carrier is symptomatic and until five to seven days post-infection (Center for Disease Control and Prevention, 2018). It may take a few days to weeks for the carrier to fully recover, but complications may arise during this recovery period. The complications that may arise are usually inflammation of the upper respiratory tree and the trachea. Respiratory complications arising due to influenza infection can develop in as little as 48 hours from the beginning of symptoms (Boktor, 2019). The virus replicates in the upper and lower respiratory passages starting from the time of inoculation and peaking after 48 hours on average (Boktor, 2019). Research has suggested that influenza A and B are different in terms of kinetics within the respiratory tract. This is what is evaluated through this study. Influenza viruses replicate in the epithelial cell lining of the upper and lower respiratory, and the pathology does not differ between natural or experimental infection (Taubenberger, 2008). This allows for the methods used in this study. Many studies have been conducted concerning the kinetics of influenza A. This study has verified that influenza A, specifically strain Cal09, infects the upper respiratory system and well as the lower. The study by Moore et al. (2014), concluded that within experimental infection, influenza A was able to infect the nasal wash and two locations in the lung. The researchers showed that the virus was able to infect a larger portion of the

lung sections as the inoculum volume was increased (Moore et al., 2014). They were able to conclude that the inflammatory response was most intense on day six following inoculation. In this study, it was verified that influenza A infection in the nasal wash peaks around 0-48 hours post infection. The titer value then showed a steady decrease as the host cleared the virus from the upper respiratory tract. Upon completion of nasal wash collection, the ferrets were euthanized, and the lungs were sampled. The titer verified that there was influenza A virus present in the lungs at this time, with little to no virus present in the nasal wash.

Similar procedures were used for the evaluation of influenza B. Less information is known about the kinetics of influenza B, making it hard to fully determine if IBV infects the lower respiratory system. The first virus used, B/Brisbane, confirmed infection of the upper respiratory system. However, unlike influenza A, the virus titer showed a peak concentration on day three, 72 hours after inoculation. The titer value then drastically decreased until little to no virus was present. Another virus, B/Phuket, was used to study infection of the lower respiratory system. B/Phuket showed nasal wash virus titers similar to Cal09 and B/Brisbane. The virus titer showed the highest on day two, but still remained quite high on day three. The titer for B/Phuket then dropped to a value indicating no viral activity around day six. Regardless of the small inconsistencies, in all three strains of influenza, the virus titer peaked around 48 hours post infection, and was no longer active in the upper respiratory tract around day five. The lung titer was then analyzed to determine the presence of B/Phuket in the lower respiratory system. There was no presence of virus in any of the four ferrets inoculated with B/Phuket. The information presented in this report confirms the kinetics of influenza A and B in the

upper respiratory system and also confirms that although influenza A infects the lower respiratory system, influenza B (B/Phuket) does not. B/Brisbane was not included in lung sampling, therefore, further research would need to be conducted to confirm the lung infectivity of this type of influenza B virus.

Conclusion

In this study, the kinetics of influenza A was verified and determined to infect both the upper and lower respiratory systems. The experiments evaluating influenza B were able to verify that the IBV infects the upper respiratory system, but no sign of lower respiratory infection was found in this study. Currently, little is known about the viral kinetics of influenza A and B during infection within an individual. World-wide influenza outbreaks can be very costly in terms of human life, infecting nearly 20-50 million people in the United States alone and causing an estimated 30-40,000 deaths per year. Influenza viruses have an extremely high mutation rate, providing a large hurdle for effective vaccinations. This constant change creates an unquestionable need for intensive basic and clinical influenza research. The goal is to recognize and anticipate how the viruses are evolving. This includes the kinetics and the replication of the virus. This thorough understanding is the first step toward creating accurate and useful antiviral therapies for influenza. As seen in the world today with COVID-19, not understanding the kinetics and infectivity of a virus can be particularly damaging to the population and economy. Influenza has been around for hundreds of years, allowing adequate time for extensive research. Research such as this is needed to keep up with the quickly evolving influenza in order to create a healthier society and economy.

REFERENCES

Anthony, K. (2018, March 26). Type B Influenza Symptoms. *Healthline*. Retrieved from https://www.healthline.com/health/influenza-b-symptoms

Baccam, P., Beauchemin, C., Macken, C.A., Hayden, F. G., & Perelson, A.S. (2006, August) Kinetics of Influenza A Virus Infection in Humans. *Journal of Virology, 80*(15), 7590-7599. doi: 10.1128/jvi.01623-05

Barnett, R. (2019) Influenza. *The Lancet,* 393(10170), 396. doi.org/10.1016/S0140-6736(19)30148-5

Belser, J. A., Katz, J. M., & Tumpey, T. M. (2011, September 1). The ferret as a model organism to study influenza A virus infection. *Disease Models and Mechanisms.* Retrieved from https://dmm.biologists.org/content/4/5/575

Boktor, S. W., & Hafner, J. W. (2019, December 5). Influenza. *PubMed*. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29083802

Borkenhagen, L. K., Salman, M. D., Ma, M.,& Gray, G. C. (2019). Animal influenza virus infections in humans: A commentary. *International Journal of Infectious Diseases,* 88, 113-119. doi.org/10.1016/j.ijid.2019.08.002

Brown, M. A., Wallace, C. S., Anamelechi, C. C., Clermont, E., Reichert, W. M., & Truskey, G. A. (2007, May 25). The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. Biomaterials, 28(27), 3928–3935. doi: 10.1016/j.biomaterials.2007.05.009

Cheng, V. C. C., To, K. K. W., Tse, H., Hung, I. F. N., & Yuen, K.-Y. (2012, April 25). Two years after pandemic influenza A/2009/H1N1: what have we learned? Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3346300/

DerSarkissian, C. (2017, November 16). Flu Statistics: What Are Your Odds of Getting the Flu? Retrieved May 4, 2020, from https://www.webmd.com/cold-and-flu/flu-statistics

Disease Burden of Influenza. (2020, April 17). *Center for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases (NCIRD).* Retrieved from https://www.cdc.gov/flu/about/burden/index.html

Dudas, G., Bedford, T., Lycett, S., & Rambaut, A. (2014, October 15). Reassortment between Influenza B Lineages and the Emergence of a Coadapted PB1–PB2–HA

Gene Complex. *Molecular Biology and Evolution*, *32*(1), 162–172. doi: 10.1093/molbev/msu287

Clayville, L. R. (2011, October). Influenza update: a review of currently available vaccines. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3278149/

Erkoreka, A. (2009, November 30). Origins of the Spanish Influenza pandemic (1918-1920) and its relation to the First World War. *Journal of Molecular and Genetic Medicine*, *03*(02). doi: 10.4172/1747-0862.1000033

Fauci, A. S., & Collins, F. S. (2012, June 22). Benefits and Risks of Influenza Research: Lessons Learned. *Science*, *336*(6088), 1522–1523. doi: 10.1126/science.1224305

Flu May Be Forever, but Vaccine Helps. (1987, December 11). Retrieved from https://www.latimes.com/archives/la-xpm-1987-12-11-vw-18686-story.html

Flu Symptoms & Complications. (2019, September 18). *Center for Disease Control and Prevention.* Retrieved from https://www.cdc.gov/flu/symptoms/symptoms.htm

Hagemann, H. (2020, April 3). The 1918 Flu Pandemic Was Brutal, Killing More Than 50 Million People Worldwide. Retrieved from https://www.npr.org/2020/04/02/826358104/the-1918-flu-pandemic-was-brutal-killing-as-many-as-100-million-people-worldwide

Hannoun, C. (2013). The evolving history of influenza viruses and influenza vaccines. *Expert Review of Vaccines,12*(9), 1085-1094. doi:10.1586/14760584.2013.824709

How Flu Spreads. (2018, August 27). *Center for Disease Control and Prevention*. Retrieved from https://www.cdc.gov/ flu/about/disease/spread.htm

Influenza hemagglutination inhibition assay. (2009, May 27). *Virology Blog.* Retrieved from https://www.virology.ws/2009/05/27/influenza-hemagglutination-inhibition-assay/

Influenza in Animals. (2018, September 27). *Center for Disease Control and Prevention.* Retrieved from https://www.cdc.gov/flu/other/index.html

Influenza. What you need to know for 2019-20. *Center for Disease Control and Prevention*. Retrieved from https://www.cdc.gov/flu/ season/faq-flu-season-2019-2020.htm#anchor_1568639330820

Kim, E.-H., Park, S.-J., Kwon, H.-I., Kim, S. M., Kim, Y.-I., Song, M.-S., … Choi, Y.-K. (2015, November 3). Mouse adaptation of influenza B virus increases replication

in the upper respiratory tract and results in droplet transmissibility in ferrets. *Scientific Reports*, *5*(1). doi: 10.1038/srep15940

Lakdawala, S. S., Jayaraman, A., Halpin, R. A., Lamirande, E. W., Shih, A. R., Stockwell, T. B., … Subbarao, K. (2015, October 1). The soft palate is an important site of adaptation for transmissible influenza viruses. *Nature*, *526*(7571), 122–125. doi: 10.1038/nature15379

Lee, C.-S., & Lee, J.-H. (2010, April). Dynamics of clinical symptoms in patients with pandemic influenza A (H1N1). *Clinical Microbiology and Infection*, *16*(4), 389–390. doi: 10.1111/j.1469-0691.2010.03117.x

Leesville Animal Hospital. (2016, June 5). What Is A Normal Temperature For My Pet? Retrieved from https://www.leesvilleanimalhospital.com/blog/2016/june/what-is-a-normal-temperature-for-my-pet-/

Matsuoka, Y., Lamirande, E. W., & Subbarao, K. (2009, May). The Ferret Model for Influenza. *Current Protocols in Microbiology*, *13*(1). doi: 10.1002/9780471729259.mc15g02s13

Miko B.A., Pereira M.R., Safdar A. (2019) Respiratory Tract Infections: Sinusitis, Bronchitis, and Pneumonia. In: Safdar A. (eds) Principles and Practice of Transplant Infectious Diseases. Springer, New York, NY. Retrieved from https://doi.org/10.1007 /978-1-4939-9034-4_20

Morris, D. E., Cleary, D. W., & Clarke, S. C. (2017). Secondary Bacterial Infections Associated with Influenza Pandemics. *Frontiers in Microbiology*, *8*. doi: 10.3389/fmicb.2017.01041

Moore, I. N., Lamirande, E. W., Paskel, M., Donahue, D., Qin, J., & Subbarao, K. (2014, December). Severity of Clinical Disease and Pathology in Ferrets Experimentally Infected with Influenza Viruses Is Influenced by Inoculum Volume. *Journal of Virology*, *88*(23), 13879–13891. doi: 10.1128/jvi.02341-14

People at High Risk For Flu Complications. (2018, August 27). *Center for Disease Control and Prevention.* Retrieved from https://www.cdc.gov/flu/highrisk /index.htm

Plaza, J. J. G., Hulak, N., Zhumadilov, Z., & Akilzhanova, A. (2016, May 5). Fever as an important resource for infectious diseases research. *Intractable & Rare Diseases Research*, *5*(2), 97–102. doi: 10.5582/irdr.2016.01009

Roberts, K. L., Shelton, H., Stilwell, P., & Barclay, W. S. (2012, August 29). Transmission of a 2009 H1N1 Pandemic Influenza Virus Occurs before Fever Is Detected, in the Ferret Model. *PLoS ONE*, 7(8). doi: 10.1371/journal.pone.0043303

Savage, G. (2003, December 6). Hemagglutinins (Haemagglutinins). *Encyclopedia of Food Sciences and Nutrition*, 3057–3060. doi: 10.1016/b0-12-227055-x/00935-4

Seladi-Schulman, J. (2019, January 2). How are Influenza A and B different. *Healthline.* Retrieved from https://www.healthline.com/health/cold-flu/influenza-a-vs-b

Smith, W., Andrewes, C., & Laidlaw, P. (1933). A Virus Obtained From Influenza Patients. *The Lancet*, *222*(5732), 66–68. doi: 10.1016/s0140-6736(00)78541-2

Taubenberger, J. K., & Morens, D. M. (2008, August 11). The Pathology of Influenza Virus Infections. *Annual Review of Pathology: Mechanisms of Disease*, *3*(1), 499–522. doi: 10.1146/annurev.pathmechdis.3.121806.154316

The Threat of Pandemic Influenza. (2005). *Institute of Medicine*. doi: 10.17226/11150

Types of Influenza Viruses. (2019, November 18). *Center for Disease Control and Prevention*. Retrieved from https://www.cdc.gov/ flu/ about/viruses/types.htm

Vaccine Effectiveness: How Well Do the Flu Vaccines Work? (2020, January 3). Retrieved from https://www.cdc.gov/flu/vaccines-work/vaccineeffect.htm

Webster, R. G., & Govorkova, E. A. (2014, May 30). Continuing challenges in influenza. *Annals of the New York Academy of Sciences*, *1323*(1), 115–139. doi: 10.1111/nyas.12462

Yang, J.-R., Huang, Y.-P., Chang, F.-Y., Hsu, L.-C., Lin, Y.-C., Huang, H.-Y., … Liu, M.-T. (2012, October 12). Phylogenetic and Evolutionary History of Influenza B Viruses, which Caused a Large Epidemic in 2011–2012, Taiwan. *PLoS ONE*, 7(10). doi: 10.1371/journal.pone.0047179

Yang, J., Lau, Y. C., Wu, P., Feng, L., Wang, X., Chen, T., … Cowling, B. J. (2018. August 24). Variation in Influenza B Virus Epidemiology by Lineage, China. *Emerging Infectious Diseases*, *24*(8), 1536–1540. doi: 10.3201/eid2408.180063

Zitzow, L. A., Rowe, T., Timothy Morken, W.-J. S., Zaki, S., & Katz, J. M. (2002, May 1). Pathogenesis of Avian Influenza A (H5N1) Viruses in Ferrets. Retrieved from https://jvi.asm.org/content/76/9/4420?ijkey=167093e8b8f83dfaf5d73bb7e28f49b0f715450a&keytype2=tf_ipsecsha