ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES IN NORTHERN CALIFORNIA

A Thesis

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Master of Science

in

Biology

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by

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Spring 2019

ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES IN NORTHERN CALIFORNIA

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ISOLATION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES IN NORTHERN CALIFORNIA

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Recent human infections with avian influenza viruses (AIV) highlight the need for continued surveillance of AIV in waterfowl. California's Sacramento Valley is an important wintering site for waterfowl from which avian influenza viruses may be isolated. To determine host correlates of AIV infection and better understand the risk posed by AIV circulating in California, we collected cloacal swabs from 2,066 hunter-killed ducks across four hunting seasons at different locations in the Sacramento Valley. We determined that Northern shovelers have a higher AIV infection rate than other waterfowl species from which we commonly sampled and that the relationship between sex and AIV infection is a poorly understood factor that needs to be investigated on a species level. Additionally, we detected three viruses of the subtype H7N3. Whole genome sequencing of these viruses revealed genetic markers that have been correlated with increased pathogenesis in mammals. All three H7N3 viruses were capable of replication in mammalian cells at levels similar to a human seasonal H1N1 virus. Additionally, at least one of these H7N3 viruses is able to cause disease in mice similarly to a

human seasonal H1N1 virus. These results provide insights into host factors of AIV susceptibility that can help guide future surveillance efforts as well as evidence of novel subtype H7N3 with mammalian adaptations that could pose a risk to public health and therefore should be monitored closely.

CHAPTER I

INTRODUCTION

Influenza viruses

Influenza viruses continually pose a significant risk to public health. For the 2017-2018 season, the United States alone saw approximately 49 million laboratory-confirmed cases, 960,000 hospitalizations, and 79,000 deaths from influenza [1]. Influenza viruses are negative-sense single-stranded RNA viruses from the Family *Orthomyxoviridae*. They can be classified into four antigenically distinct groups: A, B, C, and D. Types A and B are responsible for seasonal epidemics each year with type A frequently causing severe morbidity and mortality [2]. The influenza virus genome is broken into eight segments: hemagglutinin (HA), neuraminidase (NA), matrix protein (M), nuclear protein (NP), nonstructural protein (NS), polymerase basic 1 and 2 (PB1 and PB2), and polymerase acidic protein (PA). Characterization and naming of influenza A viruses (IAV) is based on the expression of surface proteins HA and NA, which are responsible for virus entry and exit, respectively. These surface proteins are under the greatest selection pressure relative to the remaining viral proteins as they are exposed at the virion surface and thus subject to antibody-mediated neutralization [3]. To date, 16 HA subtypes and 9 NA subtypes have been identified.

The emergence of novel IAV strains creates a larger public health issue due to the possibility of pandemic strains which cause widespread morbidity and mortality [2, 4]. Novel strains emerge through two processes, antigenic drift and antigenic shift. Antigenic drift is the gradual accumulation of random point mutations in the viral genome caused by an error-prone viral replicase enzyme which alters the way antibodies recognize and bind viral particles. This accounts for most of the genetic changes each year and is the basis for the evolution of avian

influenza viruses [5]. Antigenic shift occurs when the genomes of two or more influenza virus strains coinfecting the same cell reassort to form a new strain containing genetic information from each of the parent strains. Reassortment that results in a virus expressing a novel HA or NA protein may be highly pathogenic and have a greater potential to cause pandemics [4].

Avian influenza virus

Birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, and allies) are the natural reservoirs for nearly all IAV subtypes (16 HA subtypes and 9 NA subtypes). Avian influenza viruses (AIV) bind preferentially to α2,3-linked sialic acid receptors on target cells in the gastrointestinal tract while human-adapted influenza viruses bind preferentially to α2,6-linked sialic acids in the upper airways. Previous research and sequence analysis have demonstrated that specific amino acid changes can lead to a change in receptor specificity. In the HA protein, the Q266L mutation changes the receptor specificity from avian to mammalian in H2 and H3 subtypes while in the H1 subtype, E190D determines specificity [6]. In addition to mutations in the HA protein, temperature differences between the avian gastrointestinal tract (40°C) and human respiratory tract (32°C) greatly influence the ability of a virus to infect and replicate in various cell types [7]. Avian viruses are more adapted to the warmer temperatures in the waterfowl gut, likely preventing them from efficiently infecting cells in the human respiratory tract. These preferences typically prevent spillover of the viruses from the avian hosts to humans [8]. However, influenza viruses bearing novel HA and NA subtypes sporadically cross the species barrier and cause human infections [8]. Human infection with these novel influenza viruses of birds requires several genetic/molecular adaptations, which occur through genetic drift or genetic shift, to human cells. However, if those adaptations occur, the virus frequently causes more severe disease than what is seen with

seasonal human strains (H1N1 and H3N2) [2]. These novel viruses have HA and NA segments that the immune system does not recognize, having never previously been exposed to them, which renders any antibodies that have already been made useless. Additionally, these novel subtypes can possess any number of mutations in other gene segments that can lead to increased pathogenesis [9]. For example, mutations in the PB2 segment, along with a variety of other mutations in various gene segments, are also known to cause increased virulence and pathogenicity in mammals [8, 10]. A single mutation, E627K, in the PB2 segment has been identified and appears to be key for the virus to cause infection in humans [8]. This mutation is responsible for the ability of the virus to replicate in certain cells. With glutamic acid at position at 627, the virus has an avian preference and is unable to replicate in mammalian cells. When the glutamic acid mutates to a lysine, the virus gains the ability to replicate in mammalian cells [8]. Reassortant viruses are of great concern because of their ability to randomly acquire gene segments that already have increased fitness in mammalian cells, whether that be receptor specificity or replication ability [2].

Emergence of novel subtypes and the importance of surveillance

Several novel subtypes of avian influenza viruses have emerged to cause widespread outbreaks in economically important poultry as well as human infections [11, 12, 13, 14, 15, 16, 17]. In early 2013 the first human cases of H7N9, which causes severe lower respiratory infections, were identified in China [12]. H5N8 was first isolated from poultry in China in 2010. Since then it has spread to Europe, Africa, and North America where it continues to cause outbreaks in poultry [16]. In 2014-2015, a highly-pathogenic H5N2 caused a severe outbreak in poultry in the United States, ultimately leading to death by infection or culling of 7.4 million turkeys and 43 million chickens [18]. In early 2004, an outbreak with an H7 influenza virus

occurred in British Columbia in farmed poultry. Two workers also contracted the virus and, isolates were identified as subtype H7N3 [19].

In order to prevent such outbreaks, surveillance for avian influenza viruses with genetic or phenotypic traits that indicate adaptation to mammalian hosts is carried out by laboratories across the globe [12, 14, 15]. Given its proximity to the Pacific Flyway, a major migratory flyway, Butte County, CA provides an important location for AIV surveillance. The Pacific Flyway accounts for 20% of the waterfowl that winter in the U.S. Of the birds that migrate through the Pacific Flyway, 60% winter in the Central Valley which includes Butte County [20]. There is limited information regarding the circulation of AIVs through wild waterfowl populations in the Pacific Flyway, which is a key North American entry point for viruses due to overlap in Alaska with Eurasian flyways [21]. Our surveillance location provides an ideal point to quickly detect Eurasian lineage viruses almost as soon as they enter North America and before the health of humans or economically important poultry are threatened on a large scale.

During the 2014-2015 sampling season, we isolated over 30 avian influenza viruses from hunter-killed waterfowl. Of particular interest, we isolated three H7N3 viruses, a subtype that has been the cause of human infections in the past [13, 19]. The objective of my project was to determine what host factors influence AIV susceptibility, which subtypes of AIV are present in waterfowl in Northern California, and to characterize the risk that H7N3 viruses isolated from Butte County pose to human health using several *in vitro* and *in vivo* assays. **I hypothesized that the three novel H7N3 influenza viruses collected in Butte County would show phenotypic characteristics consistent with other mammalian-adapted viruses**. The specific aims designed to test this hypothesis were:

Specific Aim 1: Characterize the host factors that influence susceptibility to avian influenza virus infection in Butte County, CA

Specific Aim 2: Assess the replication fitness and pathogenesis of the novel H7N3 viruses through *in vitro* and *in vivo* assays.

CHAPTER II

MATERIALS AND METHODS

Sample collection and processing

Cloacal swabs were collected from 2,066 hunter-harvested waterfowl in the Colusa National Wildlife Refuge in Colusa County, CA and the Upper Butte Basin Wildlife Area (UBBWA) in Butte County, CA between October 2014 and January 2018. Waterfowl species, age, and sex were based on [22]. Viral RNA was extracted using the MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion/Applied Biosystems) and screened for the influenza virus (IAV) matrix gene by rRT-PCR. Samples with a cycle threshold (Ct) value >45 were considered negative for IAV.

Cell culture

Madin Darby canine kidney (MDCK) epithelial cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin. Normal human bronchial epithelial (NHBE) cells were maintained in bronchial epithelial cell growth medium (BEGM) supplemented with BEGM SingleQuots™ and Growth Factors (Lonza).

Virus isolation

Viruses were propagated in the allantoic cavity of 10-day-old specific pathogen-free embryonated chicken eggs at 37 °C. Allantoic fluid was collected 48-72 hours post-inoculation, cleared by centrifugation at 1500 rpm for 5 minutes, and titers were determined by $TCID_{50}$ assay on MDCK cells as described in [23].

Virus sequencing

Full genome sequencing was performed on amplified viral RNA using Superscript III high-fidelity RT-PCR kit reagents (Invitrogen), and influenza-specific universal primers (Opti1-F1– 5'GTTACGCGCCAGCAAAAGCAGG at 0.1 μM, Opti1-F2 - 5'GTTACGCGCCAGCGAAAGCAGG at 0.1 μM, and Opti1-R1 - 5'GTTACGCGCCAGTAGAAACAAGG at 0.2 μM) [24]. Samples for which the product for all eight gene segments was visually verified by agarose gel electrophoresis were purified with 0.45× volume AMPure XP beads (Beckman Coulter) and sequenced on the Illumina HiSeq 2500 platform in a single-end 100nt run format as described in [24].

In vitro infections

Cells were infected at a multiplicity of infection (MOI) of 0.01 (MDCK) or 0.05 (NHBE) for 1 h at 37°C. Cells were washed three times with PBS to remove unbound virus, and infected cells were cultured in appropriate medium (MEM for MDCK cells and BEGM for NHBE cells) containing 0.075% bovine serum albumin (BSA) and 1 μg/ml TPCK-treated trypsin. Aliquots of culture supernatants were stored at −80°C for the determination of virus titers by $TCID_{50}$ assay as described in [23].

In vivo infections

All procedures were approved by CSU Chico's IACUC and were performed in compliance with the Guide for the Care and Use of Laboratory Animals. Six- to 8-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were lightly anesthetized with isofluorane and intranasally inoculated with 50μl PBS or 10^5 $TCID_{50}$ units of the indicated viruses in 50μl PBS. Mice were monitored daily for clinical signs of infection (e.g. ruffled fur, lethargy, and decreased mobility) and weighed every 48 hours.

Statistical analyses

Statistical analyses were performed using R version 3.4.3 [25]. Chi-squared tests of equal proportion of positive rate between groups were used to determine the statistical significance of differences in AIV prevalence for comparison by year. A pairwise comparison of proportions was used to compare species against each other. A non-parametric Fishers Exact test was used to compare NOSH to other dabbling duck species across all four years. The Bonferroni method was used to adjust for multiple comparisons with both the pairwise comparison and Fishers Exact test. Chi-squared tests of association with Yates' continuity correction was used to evaluate sex differences for each species.

CHAPTER III

RESULTS

Presence of avian influenza viruses in Northern California

To evaluate which host factors are important in AIV prevalence, we analyzed surveillance data from 2,066 aquatic birds over a period of four years. There was an average of 517 samples collected each season with a high of 605 samples in 2017-2018 and low of 396 samples in 2015-2016 (Fig. 1A). Samples were analyzed by PCR for the matrix gene to determine whether or not influenza virus was present. There is an average AIV prevalence rate of 11.1% across all four years with a low of 6.7% in 2014-2015 and a high of 19.9% in 2015-2016 (Fig. 1B).

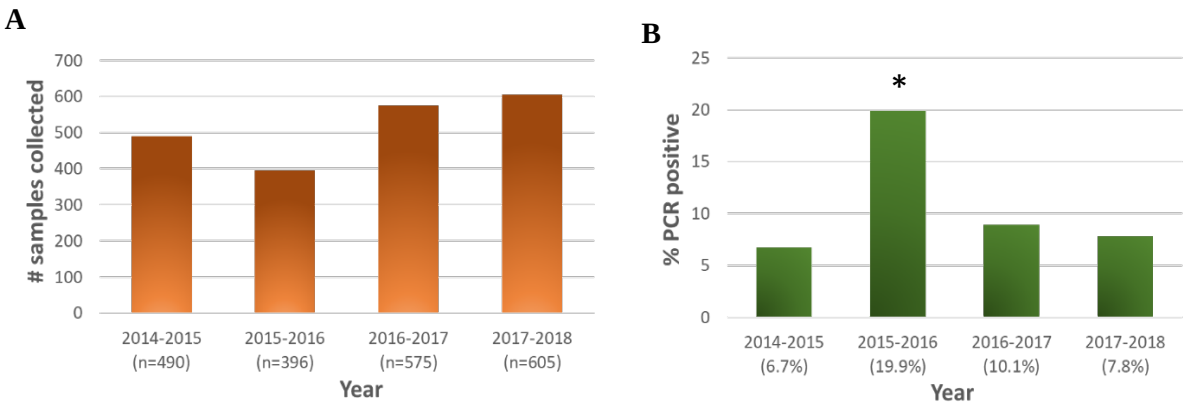

Figure 1 – Number of samples collected and percent positive by year. (A) Number of samples collected each year. (B) Percent of samples positive for avian influenza virus by year. Asterisk (*) indicates a significantly higher prevalence rate as determined by a chi-squared test of equal proportions ($p < 0.0001$).

To assess the possibility that species may influence AIV susceptibility, we first looked at overall species distribution. We have 2,066 samples from a total of 20 species of waterfowl (Table 1). Just over 90% of the samples come from the Dabbling Ducks (Anatini).

Table 1: Overview of species sampled from with number of PCR positive samples for each.

	n total	n positive
Family: Anatidae - Waterfowl		
Tribe: Anserini - Geese		
Anser caerulescens - Snow Goose (SNGO)	9	1
Anser albifrons - Greater White-fronted Goose (GWFG)	60	5
Branta hutchinsii - Cackling Goose (CAGO)	1	0
Tribe: Anatini - Dabbling Ducks		
Aix sponsa - Wood Duck (WODU)	104	1
Mareca strepera - Gadwall (GADW)	187	17
Mareca americana - American Wigeon (AMWI)	462	46
Mareca penelope - Eurasian Wigeon (EUWI)	1	0
Spatula cyanoptera - Cinnamon Teal (CITE)	27	2
Spatula cyanoptera/Spatula disccors - Cinnamon Teal/Blue-winged Teal (CITE/BWTE)*	9	2
Spatula clypeata - Northern Shoveler (NOSH)	260	54
Anas platyrhynchos - Mallard (MALL)	345	38
Anas acuta - Northern Pintail (NOPI)	159	15
Anas crecca - Green-winged Teal (GWTE)	317	18
Tribe: Aythini - Pochards		
Aythya valisineria - Canvasback (CANV)	2	0
Aythya affinis - Lesser Scaup (LESC)	1	0
Aythya collaris - Ring-necked Duck (RNDU)	89	8
Tribe: Mergini - Sea Ducks		
Bucephala albeola - Bufflehead (BUFF)	18	2
Lophodytes cucullatus - Hooded Merganser (HOME)	7	1
Tribe: Oxyurini - Stiff-tailed Ducks		
Oxyura jamaicensis - Ruddy Duck (RUDU)	2	0
Other water bird taxa		
Fulica americana - American Coot (AMCO)	6	0

*CITE/BWTE indicates that these were birds that could have been either CITE or BWTE, but we were unable to make an exact identification; females are generally considered indistinguishable

The six species with sufficient sample numbers were selected for further analysis. These species are: American Wigeon (AMWI), Gadwall (GADW), Mallard (MALL), Green-winged Teal (GWTE), Northern Pintail (NOPI), and Northern Shoveler (NOSH). The average prevalence rate of these species combined is 11.4% with individual species rates as follows: 10.5% for AMWI, 9.2% for GADW, 5.9% for GWTE, 11.0% for MALL, 10.3% for NOPI, and 21.3% for NOSH (Fig. 2A). The high prevalence rate of NOSH, nearly double the rate in other species, was an unexpected finding. To evaluate whether or not this phenomenon was a result of the overall AIV rate of 2015-2016 (19.9%), we analyzed each of the four years individually by species and found that NOSH do consistently test positive for AIV more than the other species (Fig. 2B). Sex has also been identified as a key trait that may influence AIV susceptibility [26]. However, our data suggest there may be a species dependent sext effect on AIV susceptibility as we see that female GWTE are significantly more likely to carry AIV than males (10.2% vs 2.7% respectively) (Fig. 3). We see a distribution of AIV subtypes (Fig. 4) consistent with other surveillance efforts [24, 27, 28, 29].

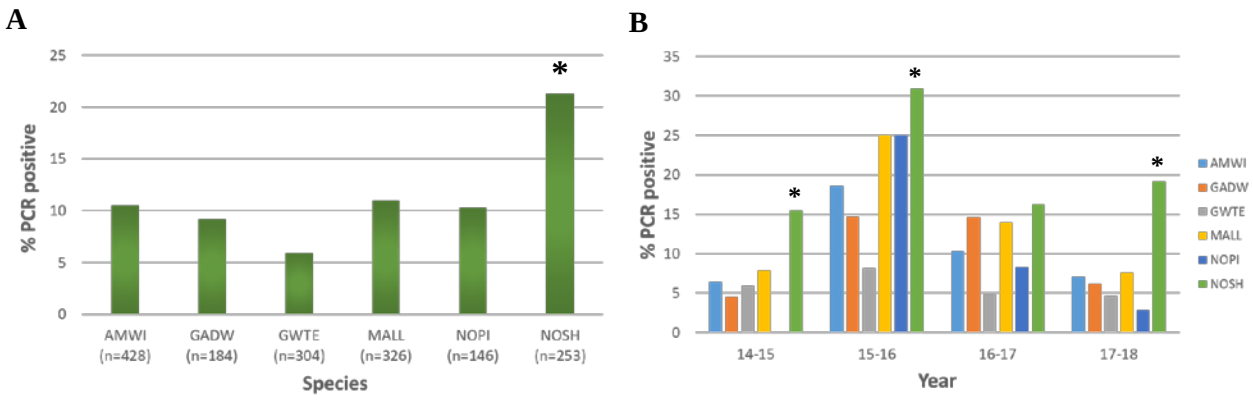

Figure 2 – Prevalence of AIV positive samples by species. (A) The number of PCR-positive swab samples from species classified as dabbling ducks is represented as % of the total number of swabs collected from that species. Asterisk (*) indicates a significantly higher prevalence rate as determined by

a chi-squared test of equal proportions (p = 0.001). (B) The number of PCR-positive swab samples from species in panel (A) broken down by year. Asterisk (*) indicates a significantly higher prevalence rate as determined by Fischers Exact Test for Count Data (p < 0.05).

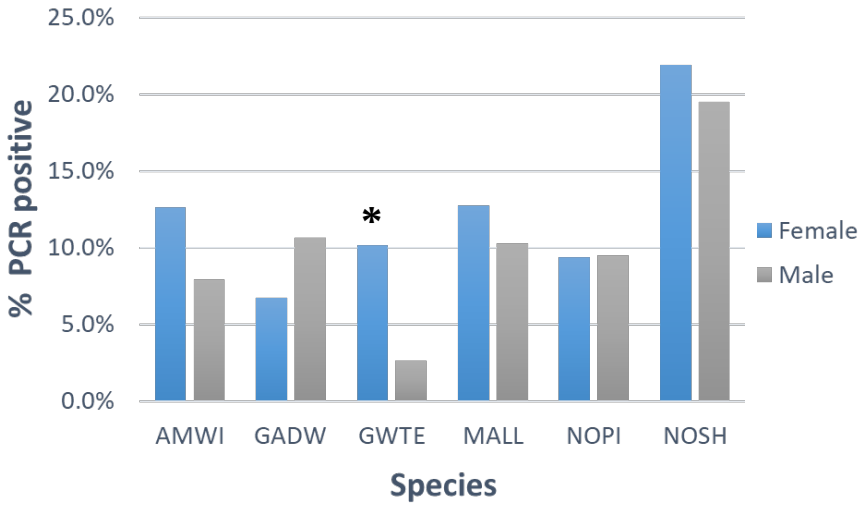

Figure 3 – Prevalence of AIV positive samples by species and sex. The number of PCR-positive swab samples from species classified as dabbling ducks is represented as % of the total number of swabs collected from that species. Asterisk (*) indicates a significantly higher prevalence rate as determined by a chi-squared test of association (p = 0.01).

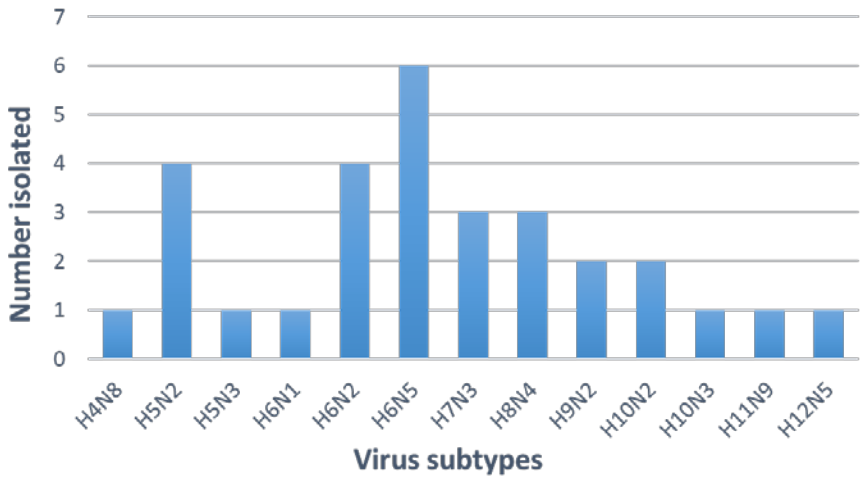

Figure 4 – Diverse HA and NA subtypes are circulating in Northern California. HA and NA subtypes of PCR + samples were determined by multi-segment RT-PCR followed by sequencing of cDNA. Complete genome sequences are available on GenBank. To date, complete genome sequences have been obtained for 30 samples. Subtypes for those samples and the frequency of detection of each sample is represented here.

In vitro replication of H7N3 viruses

Recent human infections with H7N3 viruses [13, 14, 15, 19] highlight the need for any new isolates to be evaluated for their potential to replicate in mammalian cells. Three H7N3 viruses were isolated in 2014-2015: A/mallard/California/LDC331/2015 (LDC 331), A/northern shoveler/California/LDC351/2015 (LDC 351), and A/northern shoveler/California/LDC366/2015 (LDC 366). Complete genome sequences of these viruses show several previously identified genetic signatures of pathogenesis in mammals (Table 2) [30, 31]. All three viruses have multiple mutations in the NS1, M, and PB2 gene segments that provide the ability to infect and cause disease in a mammalian host (Table 2). The PB2 mutations could prove to be key factors as they enable replication in mammalian cells [32].

Table 2: Genetic signatures present in all H7N3 viruses isolated from Butte County that are responsible for increased virulence in mammalian models

Gene	Mutation	Effect of mutation
NS1	L103F, I106M, P42S	Conferred increased virulence in mice indicated by lethality in mice and the systemic spread of infection [47, 48].
M	30D, 215A	Conferred increased virulence in mice indicated by survival rate [49].
PB2*	701D, 89V, 309D, 339K	Conferred increased polymerase activity in mouse cells [30, 50].

*Not a comprehensive list of genetic signatures present in these three viruses, the remaining mutations can be found at fludb.org

Next, the viruses were assessed for their ability to replicate in Madin Darby canine kidney (MDCK) epithelial cells, a highly permissive cell line, in comparison to influenza A/California/2009 (CA/09), an H1N1 virus known to cause human infections. After 24 and 48 hours post infection, the H7N3 viruses had comparable replication to our control H1N1 virus (Fig. 5). After confirming that the viruses in question are able to replicate in a highly permissive cell line, we needed to evaluate their ability to replicate in normal human bronchial epithelial (NHBE) cells, a primary cell line that is also the first cell type to be infected in a person coming in contact with an influenza virus. Due to limitations in NHBE cell availability, we were only able to evaluate one of the three H7N3 viruses. We observed that it has some delayed replication compared to H1N1, but is ultimately able to replicate effectively in NHBE cells (Fig. 6).

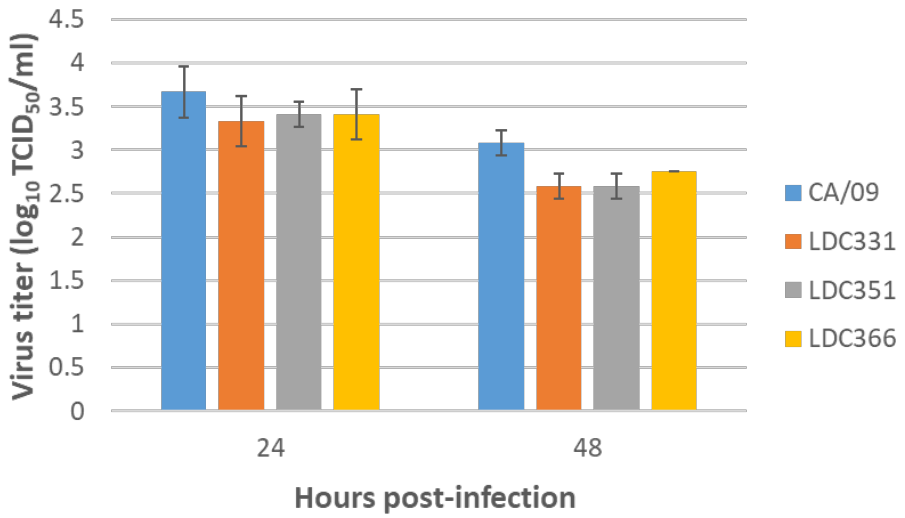

Figure 5 - *In vitro* replication kinetics of avian H7N3 influenza viruses is similar to that of a human H1N1 strain. Triplicate cultures of MDCK cells were inoculated (MOI=0.01) with influenza A/California/04/09 H1N1 (CA/09) or with three H7N3 influenza viruses isolated from wild waterfowl as part of this study (LDC331, LDC351, LDC366). At 24- and 48-hours post-infection the cell culture

supernatants were removed, and virus titers were determined by $TCID_{50}$ analysis. Error bars represent the standard error.

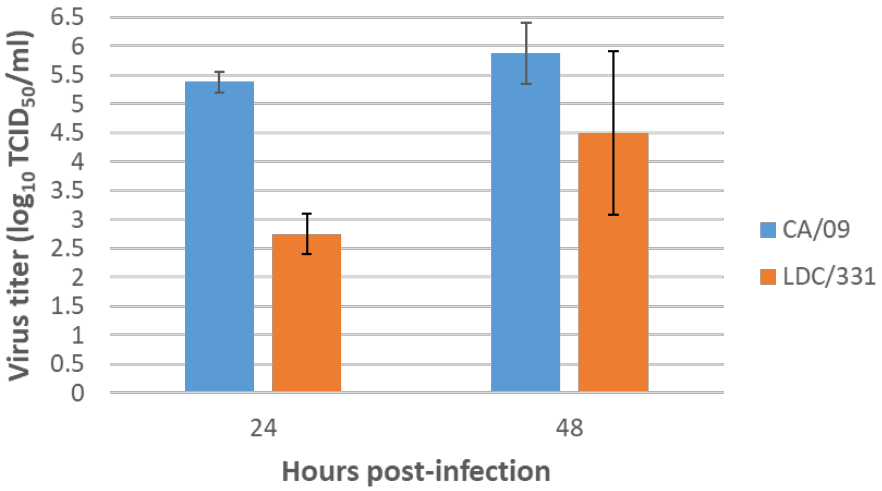

Figure 6 - *In vitro* replication kinetics of avian H7N3 influenza viruses compared to a human H1N1 strain. Duplicate cultures of NHBE cells were inoculated (MOI=0.05) with influenza A/California/04/09 H1N1 (CA/09) or LDC331 (H7N3) influenza viruses isolated from wild waterfowl as part of this study. At 24- and 48-hours post-infection the cell culture supernatants were removed, and virus titers were determined by $TCID_{50}$ analysis. Error bars represent the standard error.

In vivo replication and pathogenesis of H7N3 viruses

In addition to evaluating the ability of these viruses in mammalian cell lines, it is also important to assess their ability to infect, replicate, and cause disease in mice. In our mouse model, we were only able to evaluate two of our three H7N3 viruses and switched from CA/09 to A/New Caledonia/20/1999 (New Cal) because we found New Cal to be a more suitable, non-lethal model than CA/09 for *in vivo* experiments. Of the two H7N3 viruses tested, one of them,

LDC 351, caused minimal weight loss and signs of disease. The other virus, LDC 331, caused comparable weight loss to our control H1N1 virus (Fig. 7).

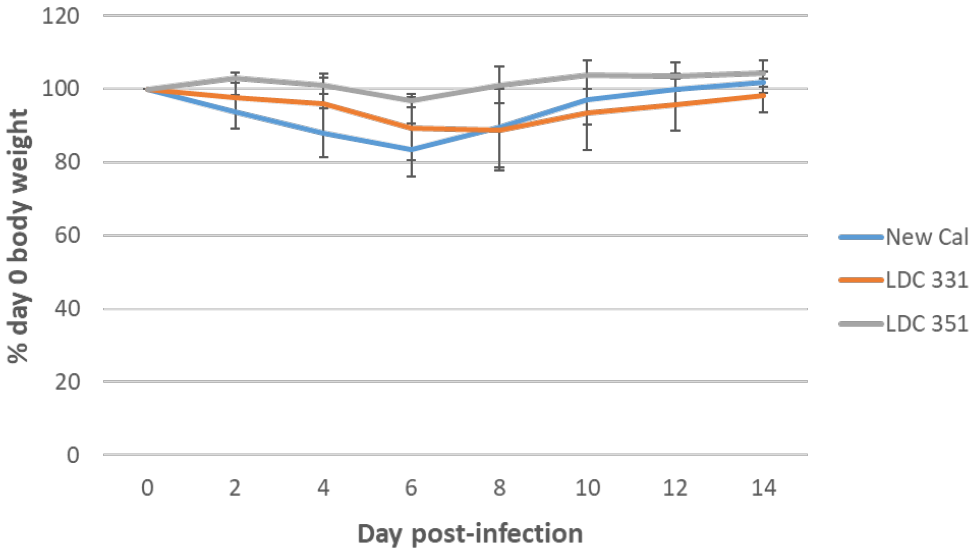

Figure 7 - H7N3 viruses have varying pathogenesis in mice. Six- to –eight-week-old female BALB/c mice were divided into groups of 10 and inoculated intranasally with PBS or 10^5 $TCID_{50}$ units of New Caledonia (H1N1), LDC331, or LDC351 H7N3 influenza viruses isolated from wild waterfowl as part of this study. Mice were monitored daily for clinical signs of infection and weighed every 48 hours. Weight loss is shown as percent of day 0 weight. Error bars represent the standard error.

CHAPTER IV

DISCUSSION

The emergence of novel strains of influenza A viruses has presented many new challenges to public health. One such challenge has been the lack of knowledge about novel strains and the threat they pose to humans. Important questions regarding host factors of AIV susceptibility and the prevalence of novel subtypes in North America still remain to be answered [2, 9, 33, 34]. This project sought to investigate both host factors influencing susceptibility to AIV infection and the potential of three novel subtype H7N3 influenza viruses from Northern California to replicate and cause disease in mammals. We identified one species of dabbling duck, NOSH, as being more likely to carry AIV than other species from which we commonly sampled (Fig. 2). Sex was also identified as a possible factor that influences susceptibility (Fig. 3), however, more work is needed to evaluate this hypothesis and specifically there is a need to investigate sex bias on a species level rather than overall.

Due to the pandemic potential of AIV, it is important to investigate any novel strains as soon as they are discovered and characterize their ability to replicate, cause disease and transmit effectively in mammals. H7N3 is one of the many novel strains that have emerged recently and have caused outbreaks in economically important poultry, as well as human infections [35, 36, 37]. H7N3 infections in humans mostly stem from contact with poultry or other infected birds, but the ability to reassort with seasonal strains could quickly lead to direct transmission leaving public health agencies poorly prepared to cope [38]. Three H7N3 viruses isolated during routine surveillance in Butte County were found to have mammalian-adaptive mutations and were able to replicate in both MDCK and NHBE cells similarly to a human-adapted H1N1 influenza virus

(Fig. 5 and Fig. 7). Additionally, at least one of the three viruses is able to cause disease in mice comparable to the human-adapted H1N1 virus (Fig. 7).

Host factors of avian influenza virus susceptibility

Ongoing surveillance efforts around the globe seek to address many of the issues scientists face with understanding the public health risks posed by influenza viruses. Antigenic drift presents constant challenges as each new mutation can lead to a virus that the human population is no longer able to recognize immunogenically [39]. Reassortment also leads to the creation of viruses that are completely new to the human immune system so even past antibodies have little to no recognition against these novel viruses [2]. In addition to tracking the viruses themselves, surveillance also allows us to study the ecology and mechanisms of AIV transmission [9, 40]. Analyzing results may provide further guidance to species, sex, or age groups that surveillance efforts should be focused on. We have collected 2,066 samples from a total of 20 waterfowl species and saw an overall AIV carry rate of about 10.5%, consistent with other surveillance groups [24, 27, 28, 29]. We observed a statistically higher prevalence rate in 2015-2016 (19.9%) compared to the other three years (Fig. 1B). This higher rate was consistent across nearly all species (Fig. 2B) demonstrating that it was not driven by any one particular species. It is also important to note that the increased prevalence in 2015-2016 was not due to a relatively high number of early season samples, which tends to produce more positives due to a large number of new immunologically naive immature birds arriving in the wintering habitat [28]. While it is not possible to determine the exact cause of this extremely high prevalence rate, one possible cause of this anomaly is the extreme drought California was in that year. This phenomenon has previously been demonstrated in West Nile virus (WNV) where researchers found a correlation between drought severity and WNV infections [41]. It would, therefore, be

beneficial to evaluate the extent to which drought conditions could influence AIV prevalence. This requires not only annual surveillance but analysis of total flooded acreage that waterfowl use in conjunction with USGS tracking of overall drought severity. This could be a vital piece of information for monitoring infectious diseases as climate change could lead to even worse drought conditions globally. Our analysis of four years of surveillance data from the Pacific Flyway has provided some insights into possible factors to focus on in future years.

The majority of our samples (90.6%) were collected from dabbling ducks (Table 2) which have a very specific feeding ecology. Of these dabbling ducks, only six species were sampled in high enough numbers to analyze statistically. Of particular interest, we showed NOSH to have an exceptionally high AIV carriage rate, approximately 21%, which is roughly 11% higher than other species sampled (Fig. 2A). There are many possible reasons for why this may be occurring including, but not limited to: the gut microbiome, physiological make-up, and the highly specialized feeding ecology of this species. NOSH are unique within the dabbling ducks due to the density of a filter-feeding structure along their bill known as lamellae. Most dabbling ducks have lamellae that have a ridge-like appearance and occur at a medium to low density, while NOSH have high-density lamellae that are structurally similar to a fine-toothed comb. This structural difference means that shovelers are eating much smaller food items than other dabbling ducks. In addition to lamellar density, their actual feeding behavior differs from dabbling ducks. In deep water, NOSH skim the surface of the water with their bills collecting zooplankton, and in shallow water, they feed off the very top layer of bottom soil, also rich in zooplankton and other invertebrates [42, 43]. Meanwhile, other dabbling ducks feed primarily on seeds, fruits, and aquatic vegetation, as their lamellae are not equipped for filter feeding. There is some evidence that zooplankton can accumulate high amounts of bacteria and viral RNA [44,

45], so it is possible that the consumption of zooplankton is causing the dramatic increase in AIV carriage in NOSH. Another possible cause for this high carriage rate is the gut microbiome of NOSH. Previous studies using MALL demonstrated that there are microbial differences in the gastrointestinal tract of AIV-positive and AIV-negative birds [46]. AIV-positive birds had much lower bacterial diversity and richness than AIV-negative birds, indicating that uninfected MALL have a more robust gut microbiome community. More research is needed to determine whether microbiome differences trigger higher susceptibility to AIV infection or if the virus itself changes the gut microbiome. Additionally, it is important to determine how these microbiome changes impact the transmission of the virus between birds [46]. In addition to the high prevalence in NOSH, previous research [26] into host factors suggest that, overall, males are more likely to carry the virus than females due to a variety of factors that lead to more frequent exposure to pathogens such as risky behavior of males (e.g. male vs. male fighting), testosterone suppresses the immune system also leading to higher susceptibility to pathogens, and lower serum antibody prevalence [26]. However, our research indicates that sex biases may be species dependent (Fig. 3).

Characterization of H7N3 viruses

During routine surveillance in 2014-2015, our lab isolated three H7N3 avian influenza A viruses from Butte County. Due to the emergence and recent human infections of H7N3 [14, 38, 47, 48], it was important to investigate the potential public health risk these viruses pose. Sequencing data (Table 2) combined with *in vivo* and *in vitro* models for influenza virus replication and pathogenesis allowed us to determine that the viruses are likely capable of infecting human cells. All three H7N3 viruses isolated in Butte County possess mutations in the M, NS1, and PB2 segments [30]. In the NS1 gene segments, all three viruses share mutations:

L103F, I106M and P42S [49, 50]. The NS1 protein is responsible for inhibiting the production of interferon (IFN), a key host response to influenza virus infection. When mutations are made at positions 103 and 106, the virus is not able to inhibit the host response as effectively which leads to drastically increased disease symptoms in mice causing higher lethality [50]. The mutation at position 42 has a similar effect as the mutations at 103 and 106 in that it also leads to increased diseases severity in mice and additionally inhibits activation of the NF-kB pathway, another key host response to viral infection [49]. The M segment of influenza viruses is crucial for assembly of virus particles and therefore replication. Mutations N30D and T215A in this segment lead to increased virulence and decreases survival in mice although more work is needed to determine the exact mechanisms [51]. The PB2 gene segment is part of the RNA polymerase complex of influenza viruses and is essential for replication of the virus. The mutation D701N proves to be a key host-range factor as this specific mutation enables the virus to replicate in and cause severe systemic infection in mice [32]. Subbarao et al. 1992 identified a key mutation in the PB2 gene, E627K, that greatly increases virulence and changes host specificity. It is important to note that this mutation is absent from all H7N3 viruses in this study, however, there is evidence that a combination of multiple other mutations can compensate for the lack of this missing E627K mutation [52]. These mutations, L89V, G309D, T339K, R447G, I495V, and A676T, are present in all three H7N3 viruses. Individually, and combined, these mutations in various gene segments led us to believe that these viruses would likely replicate in human cells and in mice.

The first step was to assess the ability of these novel H7N3 viruses to replicate in MDCK cells, a highly permissive cell line in comparison to a human-adapted virus. All three viruses had comparable replication to CA/09, a known human H1N1 influenza strain (Fig. 5). To evaluate the potential for these viruses to infect humans, we used NHBE cells as an *in vivo* model. NHBE

cells are a primary cell line taken directly from the bronchioles (upper respiratory tract) of human donors and therefore provide important clues about how the mutations in these viruses might enable them to cause human infections as this is a cell type that the virus would first encounter in a human host [53, 54]. Because all three viruses are of avian origin, there should be no receptor preference for human cells (2,3-linked sialic acids for avian viruses, 2,6-linked sialic acids for human viruses). We again compared H7N3 to CA/09 and saw delayed, but comparable replication in the NHBE cells, indicating that at least one of the three viruses may be able to infect and replicate in a human host (Fig. 6). While this is unlikely at this point in time as all known human H7N3 infections have stemmed from contact with poultry [47], it is possible for the virus to mutate or undergo reassortment with a seasonal human influenza strain and gain the ability to easily infect humans as well as the ability to transmit easily from person to person [23]. Even with a low potential for direct human infection with these H7N3 viruses, it is still interesting that they were able to infect human cells relatively easily making them part of only a handful of known viruses able to infect human cells despite having avian origins [55, 56, 57]. As mentioned previously, all three viruses possess mutations in the PB2 segment that are key for host-switching but lack any mutations in the HA protein which is responsible for receptor specificity. It is possible that, like the viruses evaluated by Oshanky et al. 2011, these H7N3 viruses are able to infect NHBE cells without much regard for receptor type, it is also possible that the PB2 mutations for host specificity may be playing a large role here, both of these factors may play a role in the replication ability, and it is possible for there to be additional factors at play that have yet to be identified.

To assess the ability of these H7N3 viruses to infect, replicate, and cause disease in mice, we infected BALB/c mice and monitored for weight loss, a marker for disease in mice, over a

two-week period. Mice are currently one of the most commonly used models for human influenza illness [58, 59]. The H7N3 viruses were again compared to a human-adapted H1N1 strain and one of the tested viruses showed comparable pathogenicity with the control virus while the second H7N3 virus we tested showed only a brief drop in weight before recovering (Fig. 7). This was an unexpected result given that all three viruses possess nearly identical mutations and were able to infect NHBE cells leading to the possibility that there may be uncharacterized differences between the three viruses that could change their virulence in the mouse model. Future work should include evaluation of LDC 351 and LDC 366 for replication ability in NHBE cells as well as *in vivo* infections with LDC 366 to assess its ability to replicate and cause disease in mice. Overall, this study highlights the need for continued surveillance efforts both in Butte County and worldwide in order to monitor the spread of H7N3 and other subtypes of influenza virus. In addition to ongoing surveillance efforts, it is also important for researchers to continue evaluating any H7N3 samples isolated from surveillance efforts for their potential to cause disease in humans.

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