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The Burden of Avian Influenza Viruses in Community Ponds in California

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This is to certify that the doctoral dissertation by

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Walden University
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Abstract

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MBA, Western Governor's University, 2011

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Dissertation Submitted in Partial Fulfillment

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Abstract

Emerging influenza viruses continue to challenge public health. The problem is public health science professionals have been battling emerging human influenza diseases with tactile and reactionary methods because there is a lack of knowledge and data at the human-animal interface. This research was a baseline study of the proportion of influenza A virus (IAV) in urban and rural communities in California. The population was artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. Surface water samples [$N = 182$] were collected from artificial recirculating ponds in California. Positivity for IAV was verified by real time RT-PCR, MDCK cells for virus infectivity, nucleotide sequencing of the RNA genome, and phylogenic analysis of IAV H5N1 strains. The proportion of IAV in rural and urban ponds favored the greater burden of IAV in urban ponds over rural ponds. The presence of waterfowl and IAV M gene sequence positivity were found not to be significantly related. The geochemical properties—pH, salinity, and water temperature at time of collection—were not predictors of IAV infectivity. This baseline research study validated these water ponds as resource sites for IAV surveillance and monitoring. The social change implications of this study can be recognized at the national and international levels, to the population level, and to the individual level by providing geospatial analysis and spatial-temporal data for IAV surveillance, initiating biosecurity measures to protect poultry industries in the United States and Brazil, and contributing to the current IAV strain library. Contributions to the IAV strain library may be used to develop vaccines against human pandemics.

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Dedication

I dedicate this dissertation to my loving wife, Christi, for supporting me each day as I traveled this journey. I also dedicate this dissertation to our three children, Emily, Oliver, and Gabrielle, for giving me a purpose to complete this journey. And lastly, I dedicate this dissertation to our parents, for providing the encouragement I needed and for just being proud of me all the way through.

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I wish to acknowledge the dissertation committee. I am very thankful to Dr. Richard Jimenez for accepting to be chair of the dissertation committee and for persevering as my adviser. His support and guidance was fundamental to my completion and success. I am very thankful to Dr. Latoya Johnson for insisting that my work be of the highest quality. I am thankful to Dr. Rodney Bowden for giving me motivating praise at the right times throughout my PhD journey. I am also thankful to the California State University Channel Islands Biology/Natural Sciences Program for providing me with a working research laboratory environment. To all my friends, colleagues, students, teachers, and advisers who guided and supported me along this journey, I cannot express my gratitude and deep appreciation fully. I am especially grateful to Catherine Hutchinson, who generously shared her meticulous research and insights that supported and expanded my own work.

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Chapter 1: Introduction to the Study

Background

The "Spanish" influenza H1N1 pandemic of 1918–1919, which caused approximately 50 million deaths worldwide, has remained an ominous warning to professionals in the field of public health (Taubenberger & Morens, 2006). Since then, new subtypes of human influenza A viruses have been detected at various times: in 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, the H3N2 subtype (Hong Kong virus) appeared; and in 1977, the H1N1 virus reappeared (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). Influenza disease emergence data are collected year-round, but economic strain on global public health to prevent and treat human influenza outbreaks has been enormous. Therefore, it is imperative to identify potential sources of the virus to help minimize outbreak occurrence.

There are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. Gaps in understanding the role of the physical and biogeochemical environment as an integral part of the influenza A viral transmission also exist (Lang, Kelly, & Runstadler, 2008). More importantly, gaps in knowledge about the burden of influenza A virus in rural and urban community settings remain present. Researchers have expected influenza risk assessment tools (IRAT) to prompt additional studies to address these key gaps in the knowledge (Trock, Burke, & Cox, 2012).

The problem in this study was that public health scientists have been battling emerging human influenza diseases with tactile and reactionary methods because there

was a lack of knowledge and data at the human-animal interface. This baseline study of the proportion of influenza A virus in urban and rural community settings may provide knowledge and biological data of significant interest at the human-animal interface. This biological data may be of significant interest for the development of IRAT.

Summary of Chapter

This chapter is a moderately detailed presentation of my dissertation study. For the benefit of the reader, the introduction of this manuscript is divided into eighteen major topic sections: research literature, gaps in the knowledge, necessity of this study, problem statement, relevance of the problem, gaps in the current literature, the purpose of the study, research questions, research hypotheses, theoretical framework, relationship of theory to study approach and research questions, conceptual framework, nature of the study, definitions, assumptions, scope and delimitations, limitations, and significance. The major topic sections; research literature, gaps in the knowledge, necessity of this study, problem statement, relevance of the problem, and gaps in the current literature are detailed with further explanation in Chapter 2. The major topic sections; purpose of the study, research questions, research hypotheses, relationship of theory to study approach and research questions, conceptual framework, nature of the study, definitions, assumptions, scope and delimitations, limitations, and significance are detailed with further explanation in Chapter 3. The chapter continues with a brief summary of the research literature related to the scope of influenza A virus.

Research Literature

Seasonal influenza virus outbreaks cause 250,000 to 500,000 deaths annually

(Keeler, 2011). The review of the literature identified many studies seeking to reduce the morbidity and mortality resulting from influenza virus outbreaks. One area of significant interest is the human-animal interface. The World Health Organization (2011) described the human-animal interface as a complex but critical juncture at which new paradigms are emerging. The aquatic virus reservoir is a human-animal interface. Franklin et al. (2011) suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals. Although several studies established the relevance of the problem, there are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses.

Ecological

Austin and Hinshaw (1984) investigated feral duck species as a source of transmission of influenza A virus and paramyxoviruses. From the investigation, Austin and Hinshaw isolated three antigenetically distinct strains of influenza A virus and two strains of paramyxoviruses. Additionally, Austin and Hinshaw suggested surveillance of healthy ducks, and the aquatic environment they frequent may be of significant interest to monitoring and controlling influenza A viruses. Austin and Hinshaw and others have identified influenza viral subtypes in various waterfowl breeds worldwide (Ferro et al., 2010; Mehrabanpour, Rahimian, Shirazinezhad, Moein, & Shayanfar, 2012). These worldwide zoonotic studies supported the hypothesis that migratory waterfowl play a role in the natural influenza virus reservoir(s) and dispersal. Similar ecological studies have

been conducted in the United States. Ferro et al. (2010) conducted one such study.

Ferro et al. (2010) focused on the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas coast. The researchers investigated the prevalence of avian influenza virus among hunter-harvested waterfowl stratified by sex, age, species. Ferro et al. identified the Blue Winged Teal (*Anas discors*), Green Winged Teal (*Anas carolinensis*), and Northern shoveler (*Anas clypeata*) as the three predominate host species for avian influenza viruses. Authors of other recent studies have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen, Franklin, Mooers, Sullivan, & Shriner, 2010; Webster et al., 1992).

VanDalen et al. (2010) were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Researchers have recognized that waterfowl fecal material and droppings are common in aquatic habitats frequented by migratory and domestic birds. The literature supported a fecal-oral transmission between waterfowl of influenza A viruses (Franklin, VanDalen, & Huyvaert, 2011). Thus, it is possible influenza viruses can be transfered between organisms via contaminated open water-sources as wetlands, lakes, and ponds. Other researchers have investigated if influenza A viruses can be detected in these contaminated aquatic habitats.

Lang et al. (2008) collected and studied sediment samples from three ponds in Alaska. The Creamer's Field Migratory Waterfowl Refuge, Alaska, is a location used by a wide variety of migratory waterfowl. The researchers collected sediment samples using a time-series approach and analyzed for influenza A virus RNA by reverse transcription-

polymerase chain reaction (RT-PCR) methodologies (Lang et al., 2008). Lang et al. demonstrated influenza viruses were readily detected in the winter when ponds were frozen, indicating the sediment could be a year-to-year reservoir of viruses to infect birds using the ponds. Zhang et al. (2006) conducted a similar a study sampling ice and water.

Zhang et al. (2006) collected and studied samples of ice or water from three northeastern Siberian lakes in the Koluma River region for the presence of influenza A virus. Analysis for avian influenza virus in the Zhang et al. study was conducted using RT-PCR methodologies. Zhang et al. found the highest frequencies of detection of influenza A virus RNAs in the lakes with the highest concentrations of migratory waterfowl, and influenza A virus RNA preserved in higher concentrations in lake ice than in lake water. Other researchers have collected and studied influenza A viruses from surface water to investigate the perpetuation between waterfowl and water-contamination (Hinshaw, Webster, & Turner, 1980; Ito et al., 1995), while others have researched the infectivity and transmission of influenza A viruses in the aquatic environment (Brown, Goekjian, Poulson, Valeika, & Stallknecht, 2009; Halvorson, Kelleher, & Senne, 1985).

In a longitudinal study, Hinshaw et al. (1980) collected and analyzed samples from waterfowl, unconcentrated lake water, and feces from lake shores near Vermillion, Alberta, Canada. The purpose of this investigation was to isolate influenza viruses and to determine whether influenza viruses continually circulated or whether the same or different strains were present from year to year (Hinshaw et al., 1980). The findings of the Hinshaw et al. study indicated the feral ducks in the study area of Canada were a perpetual reservoir influenza A viruses and paramyxoviruses. Additionally, Hinshaw et al.

asserted this longitudinal study of feral ducks would provide data on the size of the gene pool of influenza A viruses in nature and the number of viruses antigenically related to human strains present in avian species. A purpose of this dissertation was to provide data on the size of the gene pool of influenza A viruses in artificial aquatic habitats in California and the number of viruses antigenically related to human strains present in avian species that use these habitats.

In the early study by Halvorson et al. (1985), the researchers investigated the theory of water-borne transmission of avian influenza A viruses among wild waterfowl and domestic fowl. The findings of the Halvorson et al. study provided support for the theory of water-borne transmission of avian influenza A viruses among wild waterfowl and domestic fowl even though the researchers only identified certain duck-derived isolates were capable of infecting domestic turkeys. Later Ito et al. (1995) and Brown et al. (2009) further investigated the theory of water-borne transmission of avian influenza A viruses.

In the longitudinal study by Ito et al. (1995), influenza virus isolates were collected from the fecal samples of dabbling ducks and also from lake water frequented by migratory waterfowl. The results of this investigation showed the viruses remained viable in the lake water after most ducks left for migration south (Ito et al., 1995). Complementing the research by Ito et al. was the work by Brown et al. (2009). The laboratory investigation by Brown et al. indicated the pH, temperature, and salinity at levels normally encountered in nature could impact the ability of avian influenza A viruses to remain infective in water. The evidence that avian influenza A viruses can

remain infective in water is support for the theory of water-borne transmission of avian influenza A viruses among wild waterfowl and domestic fowl.

Molecular Structure

The work by Poulson (2011) and Taubenberger and Kash (2010) provided the most current detailed understanding of the molecular structure of influenza A virus. The molecular structure of influenza A virus consists of eight single-stranded RNA gene sequences. These eight single-stranded RNA gene sequences are PB2, PB1, PA, HA, NP, NA, M, and NS (Taubenberger & Kash, 2010). The methodologies to investigate and understand the molecular structure of influenza A virus include the following: virus detection by RT-PCR (Poddar, Espina, & Schnurr, 2002), virus isolation and characterization by allantoic cavities of embryonic eggs (Fouchier et al., 2005), infectivity studies using Madin Darby canine kidney (MDCK) cell lines (Brown et al., 2009), nucleotide sequencing of the RNA genome (Yamamoto et al., 2011), and phylogenic analysis using the basic local alignment search tool (BLAST) available from GenBank (Fouchier et al., 2005). Zhang, Xu, Chen, Chen, and Chen (2011) applied several molecular methodologies during their investigation of influenza A virus at the Dongting Lake wetlands.

Zhang et al. (2011) asserted the Dongting Lake wetland as an important habitat and over-wintering area for East Asian migratory birds. From the 95 water samples collected in the Zhang et al. study, an H10N8 influenza A virus was isolated, whole genome genetic sequenced, and pathogenicity investigated in mice and specific pathogen free (SPF) White Leghorn Chickens (Zhang et al., 2011). Findings of the study by Zhang

et al. were supportive for an environmental water sampling approach for influenza risk assessment. The review of the literature identified many studies and publications supporting the material and methods of this dissertation study.

A few of the studies and publications supporting the material and methods of this dissertation study included the following: Evers, Slemons, and Taubenberger (2007); Zhang et al. (2011); and Tønnessen et al. (2013). Evers et al. (2007) investigated the commercial preservative RNAlater (Qiagen) against the current method of cryo-freezing, and ethanol preservatives for influenza A virus samples. The purpose of Evers et al. investigation was to determine if using the commercial preservative RNAlater would result in improved RT-PCR amplification over the current sample preservation methods of cryo-freezing or ethanol fixation. Zhang et al. (2011) collected 200-ml water samples from areas near the habitat of migratory birds and stored the samples at -80°C until assayed. Tønnessen et al. (2013) collected samples along migratory flyways for influenza viruses, genome sequencing, and similarity analysis.

Contextual Aspects

The review of the literature identified many studies and publications seeking to reduce the morbidity and mortality resulting from influenza virus outbreaks. The researchers and scientists of these studies sought to investgate gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. The literature search strategy of this dissertation identified studies that establish the relevance of the problem and support the investigation of the burden of influenza A virus in rural

and urban ponds.

As previously noted, there are subjects within the topic of the influenza that were known and accepted in the literature. First, in the literature migratory waterfowl and shorebirds have been recognized as natural reservoirs for influenza viruses. Second, antigenic drift has been accepted as a causative process from which influenza viruses can evade humoral immunity. Third, researchers have accepted antigenic shift as a causative process which may lead to pandemic influenza viral strains. Fourth, the mixing vessel theory has been widely accepted as the process of creating novel influenza strains that are capable of host switching. Fifth, the molecular structure and the RNA genome of the influenza viron have been well documented. Sixth, the influenza virus genome database (GenBank) is available as a resource library to the international community and also a repository where new contributions can be made. However, there are subjects within the topic of the influenza that have not been well understood in the literature.

The process of antigenic shift and antigenic drift was not well understood in the literature. This process of creating novel influenza strains and subtype(s) of existing strains may result in viruses with molecular characteristics that allow for animal-to-human transmission (primary) or human-to-human transmission (secondary). The molecular characteristics that give an influenza virus the properties for animal-to-human transmission, or more seriously, human-to-human transmission were not well understood. In addition to having the properties of primary or secondary transmission, mutated influenza viruses may express varying virulence in different species.

Necessity of this Study

Modern influenza surveillance began with the World Health Organization Global Influenza Programme in 1947. Since then, many nations and international collaborations have evolved due to advancements in research and science. The influenza surveillance control programme of 1947 became the WHO Global Influenza Surveillance Network consisting of 113 national influenza centers located in 84 countries (World Health Organization, 2005). In May 2011, the Global Influenza Surveillance Network was changed to Global Influenza Surveillance and Response System. Throughout this evolution, the overarching purpose of influenza surveillance has remained the same: reduce the global burden of influenza viruses on the human population.

The annual global burden of influenza viruses on the human population has been estimated to cause 250,000 to 500,000 deaths annually (Keeler, 2011). The review of the literature identified many studies and publications seeking to reduce the morbidity and mortality resulting from influenza virus outbreaks. One area of significant interest is the human-animal interface of influenza A virus. The World Health Organization (2011) recognized the human-animal interface as a complex but critical juncture to which new paradigms are emerging. The aquatic virus reservoir is a human-animal interface. Franklin et al. (2011) suggested aquatic virus reservoirs give rise to indirect transmission. Indirect transmission may alter the transmission dynamics of influenza A virus beyond just direct interactions between infectious and susceptible individuals (Franklin et al., 2011). Investigation of aquatic virus reservoirs may reduce the annual global burden of influenza viruses on the human population.

Problem Statement

There are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. Gaps in understanding the role of the physical and biogeochemical environment as an integral part of the influenza A viral transmission also exist (Lang et al., 2008). More importantly, gaps in knowledge about the burden of influenza A virus in rural and urban community settings remain present. The problem was that public health science professionals have been battling emerging human influenza diseases with tactile and reactionary methods because there was a lack of knowledge and data at the human-animal interface. The purpose of this baseline study of the proportion of influenza A virus in urban and rural community settings was to provide knowledge and biological data of significant interest at the human-animal interface.

Emerging influenza viruses have continued to challenge public health officials. Influenza viral infections appear with such regular annual frequency that it has been common to refer to the phenomenon as *flu season* or *seasonal flu*. However, pandemic influenza events have not been regular or predictable. Taubenberger and Morens (2006) identified and provided a brief synopsis of 13 pandemic events between 1510 and 1978. Wali and Music (2011) provided a summary of public health outcomes of nine U.S. influenza epidemics that occurred between the period 1972 to 1973 through 1991 to 1992. Keeler (2011) provided a brief history of influenza pandemics from the time of discovery of the influenza virus through the last decade. Further, Keeler notably identified the sharp increase in public awareness of influenza and the potential human

health threat of the virus. Several pandemic influenza events have occurred since the early part of the 20th century. The pandemic Spanish flu of 1918 was one of the greatest human health threats.

The pandemic Spanish flu of 1918 was one of the deadliest disease outbreaks in history (Keeler, 2011; Morens & Fauci, 2007). This event was followed by the influenza pandemic of 1957-1958. The influenza pandemic of 1957-1958 first occurred in the Yunan Province of China in February of 1957 (Potter, 2001). In mid-July 1968, a widespread outbreak of acute respiratory disease occurred in Southeast China. This outbreak was reported in *The Times of London* (Cockburn, Delon, & Ferreria, 1969). Human-to-human transmission rates of this acute respiratory disease were high. By the end of July 1968, an estimated one-half a million persons had become infected (Keeler, 2011).

During the months of January and February 1976, novel swine influenza A virus outbreak occurred in Fort Dix, New Jersey. More than 230 soldiers were affected during this outbreak. Of the 230 soldiers affected, 13 soldiers (12 basic trainees and one cadre office worker) developed severe respiratory illness with one death (Gaydos, Tops, Hodder, & Russell, 2006). By the end of May and the beginning of June 1977, another outbreak of influenza was identified in Liaoning Province and Tientsin Municipality in China (Kung, Jen, Yuan, Tien, & Chu, 1978). Also known as *the 1977 Russian flu*, this outbreak is often considered the fourth pandemic of the 20th century (Keeler, 2011).

In 1996, the highly pathogenic avian influenza virus H5N1 (HPAI H5N1) emerged in southern China. This influenza A virus caused a moderate number of deaths in

geese in the Guangdong region of China (Webster, Peiris, Chen, & Guan, 2006). In February 2002, a descendant of the 1997 avian influenza A (H5N1) virus infected two persons, of whom one died (Webster et al., 2006). In late April 2005, an avian influenza A (H5N1) outbreak occurred at Qinghai Lake, China (Chen et al., 2005). This event was followed by a swine influenza emergence in Mexico.

In mid-March of 2009, Mexican health surveillance began identifying cases of influenza-like illness during a time when seasonal outbreaks were usually declining (World Health Organization, 2009a). Mexican health officials suspected these phenomena to be an outbreak of a newly emerged influenza virus (World Health Organization, 2009b). Two months following, the newly emerged influenza virus appeared in the United States. By mid-April 2009, two children in Southern California developed febrile respiratory illness caused by infection from genetically similar swine virus, a novel influenza A (H1N1; Centers for Disease Control and Prevention, 2009). The novel influenza A (H1N1) virus rapidly achieved global spread. By the end of May 2009, nearly 13,000 novel influenza A (H1N1) virus infection cases had been reported in over 40 countries (World Health Organization, 2009b). The 2009 H1N1 reference vaccine virus selected by the WHO was the A/California/7/2009 (Centers for Disease Control and Prevention, 2010). On August 10, 2010 the WHO Director-General Dr. Margaret Chan announced the H1N1 influenza event had moved into the postpandemic period (World Health Organization, 2010).

At the time of this writing, the current WHO phase of pandemic alert for avian influenza A (H5N1) was ALERT (World Health Organization, 2013). The potential for

further spread of avian influenza A (H5N1) virus and the high case fatality rate warrants further surveillance. International scientific collaboration would suggest pandemic influenza viruses may arise months prior to their emergence in humans, hence providing a window for the identification and implementation of preparedness and response activities before a pandemic occurs (World Health Organization, 2011). Artificial water ponds in rural and urban communities may be significant habitats for environmental surveillance for influenza A viruses.

Relevance of the Problem

Scholars have known and accepted certain subjects within the topic of the influenza. Researchers recognized migratory waterfowl and shorebirds as natural reservoirs for influenza viruses. The phenomenon of antigenic drift was accepted as a causative process from which influenza viruses could evade humoral immunity. Further, researchers accepted the phenomenon of antigenic shift as a causative process that may lead to pandemic influenza viral strains. Mixing vessel theory has been widely accepted as the process of creating novel influenza strains that are capable of host switching. The molecular structure and the RNA genome of the influenza viron have been intensively studied and well documented. Lastly, the influenza virus genome database (GenBank) is a resource library to the international community and a repository where new contributions can be made and data shared. However, there are subjects within the topic of the influenza that were not well understood in the literature.

Gaps in the Current Literature

A unique and problematic property of influenza viruses is their ability to evade host immune responses. The evolutionary traits of antigenic drift and antigenic shift give influenza viruses a "natural fluid" presence resulting in seasonal outbreaks (Keeler, 2011). Keeler (2011), along with others, asserted that the emergence of novel influenza viruses through antigenic drift or antigenic shift can result in significant increases in morbidity and mortality during any given influenza season.

In contrast to seasonal influenza viruses, pandemic strains are the result of major antigenic changes in the HA or NA protein known as antigenic shift (Matthews & Orman, 2011). These antigenic shifts may result in novel combination of HA or NA. The novel influenza virus may spread among the immunologically naive human population (Matthews & Orman, 2011). The sequence of the emergence of a novel influenza virus and an immunologically naïve human population may lead to a pandemic.

Potter (2001) asserted two conditions be met for an outbreak of influenza to be classed as a pandemic: firstly, the outbreak of infection, arising in a specific geographical area, spreads throughout the world; a high percentage of individuals are infected resulting in increased mortality rates. Secondly, a pandemic is caused by a new influenza virus A subtype, the HA of which is not related to that of influenza viruses circulating immediately before the outbreak, and could not have arisen from those viruses by mutation (p. 574). Investigation into the molecular mechanisms of influenza A viruses was prominent in current literature. This was likely due to recent advances in molecular and genetic sciences.

As previously noted, the process of antigenic shift and antigenic drift was not well understood by current researchers. This process of creating novel influenza strains and subtype(s) of existing strains may result in viruses with molecular characteristics that allow for animal-to-human transmission (primary) or human-to-human transmission (secondary). This process is consistent with the assertion by Potter (2001) that a pandemic is caused by a new influenza virus A subtype, the HA of which is not related to that of influenza viruses circulating immediately before the outbreak, and could not have arisen from those viruses by mutation. The molecular characteristics that give an influenza virus the properties for animal-to-human transmission, or more seriously, human-to-human transmission, were not well understood. In addition to having the properties of primary or secondary transmission, mutated influenza viruses may express varying virulence in different species.

The determinants of influenza virus virulence were not well understood. The determinants of influenza virus virulence among migratory waterfowl need further investigation. As previously noted in this chapter, an infected asymptomatic migratory waterfowl may host and disperse a highly pathogenic influenza strain along the migratory flyways. This is consistent with the theory of water-borne transmission by Webster et al. (1992) that migratory waterfowl introduce influenza virus to a water pond from fecal contamination. Further, there has been a lack of understanding of the association of which species of waterfowl can host which influenza viral strains and cause viral shedding along which migratory flyways and watershed breeding grounds. To investigate this conundrum, geospatial analysis and spatial-temporal distribution studies may be of

significant interest. In this dissertation, I attempted to fill the gap in the knowledge of the geographical dispersal of influenza A viruses in aquatic habitats using geospatial analysis.

Purpose of the Study

At the time of this writing, no known research was underway to investigate and compare the proportion of influenza A virus in recirculating artificial ponds in rural and urban geographical locations. A review of current literature did not identify any other studies that attempted to collect influenza A virus data from artificial water ponds in rural or urban communities. Several studies investigated the presence or persistence of influenza viruses to natural waterfowl wetlands (Austin & Hinshaw,1984; Ferro et al.,2010; Lang et al., 2008). The purpose of this quantitative study was to extend the previous environmental virology research of influenza in natural waterfowl wetlands to rural and urban communities.

The role of the aquatic virus reservoir as a human-animal interface has not been fully understood. The purpose of this dissertation study was to investigate the aquatic virus reservoir. Three constructs support the investigation of the aquatic virus reservoir. First, the aquatic virus reservoir is a human-animal interface. Second, researchers have suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals (Franklin et al., 2011). Third, researchers have recognized the human-animal interface as a complex but critical juncture at which new paradigms are emerging (World Health Organization, 2011).

For this quantitative dissertation study, I collected and analyzed primary data. The epidemiological triangle was the framework for the primary data to be collected. The epidemiological triangle has three vertices: host, agent, and environment. The *hosts* are the organisms harboring the disease. The *agents* are capable of causing the disease. The *environments* are where the hosts and the agents can interact. For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) (Franklin et al., 2011). The agents are influenza A viruses. The environments are the natural and artificial habitats where the hosts are found.

The independent environmental variables of this study were the geographical locations of the artificial water ponds and geochemical properties of the artificial water ponds. The geographical location data included rural or urban community, latitude and longitudinal coordinates, altitude, and approximate water pond surface area. The geochemical properties of the artificial water pond sample measure included pH, salinity, and temperature. Additionally, the presence or absence of waterfowl at the time of sample collection was included as an independent host variable.

The dependent agent variables were influenza A viruses. Influenza A virus data included detection for M gene by RT-PCR using World Health Organization recommended primer sequences, infectivity by hemagglutination assay using MDCK cell line, and gene sequencing for H5N1 influenza A virus using World Health Organization recommended primer sequences at Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California).

Research Questions

Research Question 1 (R1): Is there a difference in the burden of influenza A virus in rural ponds compared to urban ponds?

Research Question 2 (R2): Is there an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds?

Research Question 3 (R3): Is there an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds?

Research Question 4 (R4): Are H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds novel gene sequences or have the gene sequences been previously identified elsewhere?

Research Hypotheses

Null Hypothesis 1 ($H_0 1$): The burden of influenza A virus in community ponds has no association to geographical location.

Alternative Hypothesis 1 ($H_a 1$): The burden of influenza A virus in community ponds is associated to geographical location.

In this study the proportion and probability of the presence of influenza A virus were investigated in recirculating artificial ponds in rural and urban geographical locations. Rural ponds were viewed as one population and urban ponds as another population. The dependent agent variables were influenza A viruses.

Null Hypothesis 2 ($H_0$2): There is no association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

Alternative Hypothesis 2 (H_a2): There is an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

In this study, rural community ponds were viewed as one population and urban community ponds as another population. The comparison of these two populations included proportion of influenza A viruses. The independent environment and host variables were as follows: latitudinal and longitudinal coordinates, altitude, approximate water pond surface area, and the presence of absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) at the time of sample collection. The dependent *agent* variables were influenza A viruses.

Null Hypothesis 3 ($H_0$3): There is no association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

Alternative Hypothesis 3 (H_a3): There is an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

Brown et al. (2009) investigated the infectivity of 12 low pathogenic avian influenza viruses across various pH, salinities, and temperatures in a biosafety level 2 laboratory setting. In this dissertation study, I investigated if the findings of the Brown et al. study were consistent in rural and urban community pond samples. The independent

variables were the geochemical properties of the artificial recirculating water ponds: pH, salinity, and temperature. The dependent agent variables were the infectivity of isolated influenza A viruses. Influenza A virus infectivity were conducted by hemagglutination assay using MDCK cell line. The utilization of MDCK cell line to determine infectivity of influenza A viruses has been used by others (Brown et al., 2009).

Null Hypothesis 4 ($H_0$4): The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are not novel gene sequences and the gene sequences have been previously identified elsewhere.

Alternative Hypothesis 4 (H_a4): The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are novel gene sequences and the gene sequences have not been previously identified elsewhere.

In this study, water samples were collected directly from rural and urban ponds. The analysis for the presence of influenza A virus involved commercially available influenza A/B viral RT-PCR detection kits suitable for water analysis. World Health Organization recommended RT-PCR primers be used for influenza A virus H5N1 gene sequencing. The laboratory analysis for the presence of influenza A virus and influenza A virus infectivity were conducted in a biosafety level 2 laboratory at the California State University Channel Islands campus. Water samples positive for H5N1 influenza A viral strains were submitted for gene sequencing analysis to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California). The H5N1 influenza A virus gene sequencing results were compared to the influenza A viral gene sequence database GenBank (National Institute of Health genetic sequence database).

Theoretical Framework

The theoretical framework for this study was based on the hypotheses proposed by Webster et al. (1992) and others that (i) migratory waterfowl are the natural reserve of influenza viruses, and (ii) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. Adding to the theoretical framework was Scholtissek et al.'s (1985) mixing vessel theory. Mixing vessel theory posits the viral adaptation from host animal to human infectivity occurs in an intermediate host animal such as a swine or waterfowl (Ma, Kahn, & Richt, 2009).

Relationship of Theory to Study Approach and Research Questions

Water analysis has been an important scientific approach to understanding the epidemiological triangle model of influenza viral diseases. Ferro et al. (2010) investigated and analyzed host species for the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas coast. Other researchers have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen et al., 2010; Webster et al., 1992). VanDalen et al. (2010) investigated and analyzed influenza virus agents from waterfowl. VanDalen et al. were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Thus, it is possible that influenza viruses can be detected in open water-sources as wetlands, lakes, and ponds.

Study Approach

For this quantitative dissertation study, I collected and analyze primary data. The epidemiological triangle provides framework for the primary data collected. The

epidemiological triangle has three vertices: host, agent, and environment. The host is the organism harboring the disease. For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) (Franklin et al., 2011). The agents are the influenza A viruses. The environments are the natural and artificial habitats where the hosts are found.

The proportion and probability of the presence of influenza A virus were investigated in recirculating artificial ponds in rural and urban geographical locations. I attempted to isolate influenza A virus from the water samples collected. The detection of influenza A viruses in the collected water samples were support for the hypotheses proposed by Webster et al. (1992) and others that (i) migratory waterfowl are the natural reserve of influenza viruses, and (ii) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. Further, the detection of influenza A virus in the collected water samples were used to analyze the difference in the burden of influenza A virus in rural and urban community ponds.

The environmental factors; geographical location, altitude, estimated surface areas, and the observed presence of waterfowl were investigated as independent variables to the outcome of influenza A virus detection. I attempted to answer if there is an association between environmental factors and the presence of influenza A virus. An association between environmental factors and the presence of influenza A virus may allow for the generalization that certain environmental factors are associated to influenza A virus infected waterfowl.

The biochemical properties of pond water; pH, salinity, and temperature were investigated as independent variables to the outcome of infectivity of influenza A virus. Brown et al. (2009) conducted a similar study. I attempted to apply the laboratory findings of the Brown et al. study to artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. An association between biogeological factors of pond water and influenza A virus infectivity may allow for the generalization of certain biogeological properties of pond water as risk factors at the human-animal interface.

Isolated H5N1 influenza A virus samples were submitted to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California) for gene sequencing. I will used the gene sequencing data to investigate if the isolated H5N1 influenza A viruses are novel or have been previously identified elsewhere. The detection of various subtypes of H5N1 influenza A virus may allow for generalization of the phenomena of antigenic drift and the Mixing Vessel Theory. Mixing Vessel Theory posits the viral adaptation from host animal to human infectivity occurs in an intermediate host animal such as a swine or waterfowl (Ma et al., 2009). The detection of novel H5N1 influenza A viruses may be of significant interest to the scientific community.

Conceptual Framework

The fundamental question of how avian influenza viruses switch hosts remains unanswered. Additionally, it is poorly understood how the viral genetic changes effect human adaptation, human-to-human transmissibility, and pathogenesis (Taubenberger & Kash, 2010). To further understand the characterization of influenza viruses, some

researchers have approached the topic of avian influenza through the lens of the epidemiological triangle. A more detailed analysis of the epidemiological triangle as it pertains to influenza A viruses and this research is included in Chapter 2.

Nature of the Study

Water analysis has been an important scientific approach to understanding the epidemiological triangle model of influenza viral diseases. A constellation of studies have investigated influenza A virus using water analysis. Ferro et al. (2010) isolated and identified the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas Coast. Ferro et al. (2010) identified the Blue Winged Teal (*Anas discors*), Green Winged Teal (*Anas carolinensis*), and Northern shoveler (*Anas clypeata*) as the three predominate host species for avian influenza viruses. Other researchers have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen et al., 2010; Webster et al., 1992). VanDalen et al. (2010) were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Other researchers have investigated if influenza A viruses can be detected in these contaminated aquatic habitats (Lang et al., 2008; Stallknecht & Brown, 2009; Zhang, Shoham, Gilichinsky, Davydov, Castello, & Rogers, 2006). Zhang et al. (2006) investigated frozen lake water as an abiotic reservoir for influenza viruses.

Zhang et al. (2006) collected samples of ice or water from three northeastern Siberian lakes in the Koluma River region. Influenza A viruses were isolated and analyzed using reverse transcription-polymerase chain reaction (RT-PCR)

methodologies. Other researchers have studied influenza A viruses in surface water samples to investigate the perpetuation between waterfowl and water-contamination (Hinshaw et al., 1980; Ito et al., 1995), while others have researched the infectivity and transmission of influenza A viruses in the aquatic environment (Brown et al., 2009; Halvorson et al., 1985). These studies support the theory that influenza viruses can be transfered between organisms via open water-sources as wetlands, lakes, and ponds. A more detailed analysis of investigating influenza A virus using water analysis is included in Chapter 2.

Research Approach

In this dissertation study, I upheld society's trust that scientific research results are an honest and accurate reflection of a researcher's work (National Academy of Sciences, National Academy of Engineering, and Institute of Medicine of the National Academies, 2010). The primary data analyses were quantitative using a cross-sectional approach comparing proportions, bivariate and multivariate logistic regression analysis of recirculating artificial ponds in rural and urban geographical locations. The independent variables included: geographic community location (rural or urban), GPS location (latitude and longitude), altitude, approximate water pond surface area. The dependent variables included: influenza A virus detection, influenza A virus infectivity endpoint titer, influenza A virus (H5N1) characterization. Variables evaluated as possible mediators and moderators included: water pH at collection, water salinity at collection, water temperature at collection, and presence or absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*).

Sampling and Data Analysis

A convenience sampling approach was used. The geographical area was the state boundaries of California. Equal sample sizes from rural and urban communities were attempted. A representative sampling from each of the 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l) were attempted. The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban communities.

Water samples were collected as previously published in the *U.S. EPA Field Sampling Guidance Document #1225*. A dip sampler for water collection was used. A dip sampler is useful for situations where a sample is to be recovered from an outfall pipe or along a pond bank where direct access is limited. The long handle on such a device allows access from a discrete location so as not to disturb wildlife (U.S. Environmental Protection Agency, 1999). The water volume per sample was 200 ml. Other researchers have collected 200 ml volume water samples for influenza studies (Zhang et al., 2011).

The statistical analysis software was IBM SPSS Statistics 21 and Microsoft Office Excel 2007. Geographical graphing and mapping software were Google Earth. Data integrity was maintained by recording data and observations with ink into field notebook and laboratory notebook. Hand written entries were transferred or transcribed to Microsoft Office Excel 2007 Spreadsheets. All electronic data had digital backup and was password protected. Since I collected primary data, data cleaning and screening

procedures were not necessary. A more detailed explanation of the materials and methods of this dissertation are included in Chapter 3.

Definitions

Several not commonly used terms-outside of the topic of influenza-are necessary for this dissertation. The manuscript by Reid, Taubenberger, and Fanning (2004) includes definition of these terms.

Antigenic drift: Minor changes in viral antigens due to gradual accumulation of mutations over time.

Antigenic shift: Sudden change in viral antigens due to acquisition of one or more novel surface-protein-encoding genes by the process of reassortment.

Clade: Traits (for example, sequences) that form a distinct group on phylogenetic analysis.

Non-synonymous substitution: Nucleotide substitution in a codon that results in an amino acid replacement.

Nucleotide sequences: The genome of the influenza A virus consists of eight RNA segments coding for 10 different proteins.

Parsimony analysis: A type of phylogenetic analysis in which many possible trees are compared to find the tree requiring the fewest evolutionary changes.

Phylogenetic analysis: Analysis of the evolutionary connections between traits (for example, sequences).

Reassortment: Due to the segmented nature of the influenza A virus genome (eight individual RNA segments), influenza viruses can undergo a process of genetic

reassortment to produce new variant strains of virus. In a cell infected with two different influenza A virus strains, gene segments from each can be packaged into viable hybrid virus strains.

Subtype: A designation for influenza A viruses describing the antigenic group to which the two dominant surface glycoproteins — haemagglutinin (HA) and neuraminidase (NA) — belong, written in the form HXNX, wherein one of the 16 possible HAs and one of the nine possible NAs is listed, for example, H1N1 or H3N2.

Synonymous substitution: Nucleotide substitution in a codon that does not result in an amino acid replacement. These terms are necessary and used throughout this dissertation for clarity.

Assumptions

There are several assumptions about influenza A viruses that are believed, but cannot be demonstrated to be true. Firstly, the unique and problematic property of influenza viruses to evade hosts immune responses. Secondly, the phenomena of human-animal transmission are not well understood. It is possible the ability to evade host immune responses and the human-animal transmission are the evolutionary traits that give influenza viruses a “natural fluid” presence resulting in seasonal outbreaks. Keeler (2011) and others asserted the emergence of novel influenza viruses through antigenic drift or antigenic shift can result in significant increases in morbidity and mortality during any given influenza season. The outcome of antigenic drift or antigenic shift are routinely identified, however the mechanism of the processes are insofar only theorized.

A pandemic is the outbreak of infection, arising in a specific geographical area and spreads throughout the world resulting in a high percentage of infected individuals and resulting in increased mortality rates (Potter, 2001). It has been assumed that pandemics may be caused by a new influenza A virus subtypes. The new influenza A virus subtype may have an HA of which is not related to that of influenza viruses circulating immediately before the outbreak, and could not have arisen from those viruses by mutation (Potter, 2001). The 1918 Spanish flu is assumed to be caused by a "new influenza A virus" subtype. Investigation into the influenza virus responsible for the 1918 Spanish flu has not yet been fully discovered. Investigation into the molecular mechanisms of influenza A viruses is prominent in current literature. This may be due to recent advances in molecular and genetic sciences.

Artificial water ponds in rural and urban communities are sites of the human-animal interface. The investigation of the burden of influenza A virus in artificial rural and urban ponds may be of significant interest to the scientific community. In this dissertation study, I attempted to investigate the burden of influenza A virus in artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. The geographical sampling region for rural and urban ponds was the eastern Pacific flyway for migratory birds in California. For this study, it was necessary to make the assumption that artificial water ponds in rural and urban communities are sites of the human-animal interface because of fecal shedding and contamination from influenza A virus infected waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*). I attempted to isolate and investigate influenza A viruses in samples collected from water

ponds in rural and urban Californian communities. My investigation included infectivity titers and subtyping of influenza A virus (H5N1) strains. Infectivity and subtyping of isolated influenza A viruses may identify strains previously known capable for animal-human transmission, or human-human transmission.

Scope and Delimitations

A specific aspect of this dissertation is the isolation and characterization of influenza A virus (H5N1). To date, avian influenza A (H5N1) has not shown to be effectively transmitted from person to person, however, the high case fatality rate in Southeast Asia is alarming (Yuen & Wong, 2005). Ungchusak, et al. (2005) asserted the reassortment events of avian influenza A (H5N1) since its emergence in China in 1997, suggests the virus may become more efficient in infecting humans, either by acquiring genetic material from a human influenza virus through reassortment or by adapting its receptor binding sites.

Populations Included

Artificial water ponds in the geographic locations of rural and urban communities are sites of the human-animal interface. The target population for this dissertation is artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. There are 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l). The population size of artificial recirculating water ponds in California is unknown.

Populations Excluded

Excluded from this study were natural ponds and non-recirculating artificial water environments. Natural bodies of water may be the result of in-flows and out-flows of water in combination with geographical contours. The in-flow and out-flow of water will have a rate of water change-over which cannot be addressed in the analysis of the data. Similarly, non-recirculating artificial water environments will have a rate of water change-over which cannot be addressed in the analysis of the data. Also excluded from this study was the Gravity model as applied to infectious disease dynamics.

Gravity Model

The Gravity models of population movements can be traced back the work by Zifp in the 1940s (Truscott & Ferguson, 2012). Truscott and Ferguson (2012) investigated gravity models to fit human mobility movements as they applied to epidemic modelling. Jandarov, Haran, Bjornstad, and Grenfell, (2013) investigated a novel gravity model for infectious diseae dynamics. The understanding of disease dynamics may be of significant interest in the management and with pressing disease issues such as disease emergence and epidemic control strategies (Jandarov, Haran, Bjornstad, & Grenfell, 2013). In both these studies, the probabilistic models for epidemic management require known human population behaviors.

To investigate gravity model as it pertains to influenza A viruses in nature, will require a greater understanding of the population behaviors of migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*). For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) (Franklin

et al., 2011). Long-range migratory patterns of waterfowl may explain the global dispersion of highly pathogenic avian influenza H5N1 virus (HPAI H5N1) (Gaidet et al., 2010). Migratory bird population data would be necessary to understand population behaviors. However, Link and Sauer (2002) are critical of the validity of North American Bird Breeding Survey population data. Link and Sauer reasoned the flight patterns of the certain migratory waterfowl are not compatible with the observation methods of North American Bird Breeding Survey. Thus, population studies of migratory birds are not a reliable influenza disease surveillance tool and would not provide reliable data for gravity modeling for influenza disease dynamics in nature.

Generalizability

The potential generalizability of this study is to the artificial recirculating water ponds located in rural and urban Californian communities. This generalizability may be the findings of the analysis of environmental factors: rural or urban community, latitude and longitudinal coordinates, altitude, and approximate water pond surface area. However, the findings of the analysis of geochemical properties of water pond samples; pH, salinity, and temperature may be generalizable beyond the target populations of this dissertation.

Limitations

There are two threats to the external validity of this study. The first threat to external validity is centered to the theoretical framework of this dissertation. The theoretical framework for this study were based on the hypotheses proposed by Webster et al. (1992) and others that (a) migratory waterfowl are the natural reserve of influenza

viruses, and (b) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. The geographical area was the state boundaries of California. Thus, the findings of this dissertation are limited to geographical locations along the migratory flyways of the waterfowl within the boundaries of California.

To address this threat to the external validity of this dissertation study, the sampling and data analysis plan are presented in a fashion so the study may be reproduced accurately by others using the same or different geographical locations. The second threat to external validity is a result of the inclusion criteria.

Recirculating artificial water ponds require mechanical equipment for recirculation. This study does not include the type or design of the mechanical equipment used for recirculation as an independent variable. Thus, the mechanical equipment used for recirculation cannot be evaluated as a mediator or moderator of the outcome variables of this dissertation study. However, estimated surface area of the recirculating artificial water ponds as an independent variable may be of significant interest to future studies seeking to investigate factors that associate to the burden of influenza A virus at the human-animal interface in communities.

The primary data collection of dissertation study is heavily weighted by laboratory instrumentation and molecular analysis. There are two threats to the internal validity of this dissertation study: instrumentation and laboratory assays. Firstly, inaccuracy of the instrument used for this study may systematically alter the data.

Secondly, inaccuracy of the laboratory assays may fail to accurately detect influenza A virus. To address the possible inaccuracy of the instrumentation, the same instruments were used on all samples. Thus, errors, if identified, may be corrected across all samples equally. To address the possible inaccuracy of the laboratory assays, positive and negative control samples were to be processed simultaneously as study samples. The positive control for influenza A virus was not obtained. The negative control used for influenza A virus was sterile normal saline. Threats to statistical conclusion validity have not been identified.

Significance

At the time of this writing, no known research is underway to investigate and compare the proportion of influenza A virus in artificial rural and urban ponds. The discovery of the proportion of influenza A virus in rural and urban communities will establish baseline measures for future monitoring and impact studies in these settings. These baseline measures may initiate surveillance data for IRAT programs.

Even though the IRAT is not designed to predict the next pandemic, IRAT is expected to identify gaps in the knowledge, prompt further research, assist with clear documentation of the thought process, and focus risk management efforts (Trock et al., 2012). The development of baseline measures of the environmental burden of influenza A virus in communities will inaugurate new data collection for IRAT.

Contributions of the Study

There are four topic areas where in this dissertation study, I attempted to fill gaps in the knowledge and extend the knowledge in the discipline. First, I attempted to fill a

gap in the knowledge about the human-animal interface of influenza in aquatic habitats. I attempted to investigate the burden of influenza A virus in rural and urban artificial recirculating water ponds. Using a cross-sectional study approach, I attempted to apply geospatial analysis to record baseline measurements of the presence of influenza A viruses for future surveillance. Future influenza surveillance methods may use the data from this study to develop spatial-temporal monitoring for influenza viruses. Second, the materials and methods of this study may be used for future influenza surveillance. Advantages of the aquatic sampling method and analysis of this study over current practices are: no live bird capture, no hunter harvest, and no fresh fecal sampling from waterfowl. Limitation of live bird capture is only being able to capture birds that are present at the same time as the researchers. Limitation of hunter harvest is not sampling non-game birds. Limitation of fresh fecal sampling from waterfowl is only being able to sample birds that are present at the same time as the field researchers. Third, the geospatial analysis of this dissertation resulted in data that may be useful for future investigations of the gravity model of infectious disease dynamics as it applies to influenza A viruses. These future studies may seek to investigate possible association between influenza A virus contaminated aquatic habitats and human influenza infections. Fourth, I attempted to investigate the association of geographical and environmental features of aquatic habitats to the burden of influenza A virus at the human-animal interface. To add to the conversation of knowledge of influenza disease, this dissertation will build upon the materials and methods of others.

Water analysis for influenza virus has been conducted in the natural environment of Alaskan (Lang et al., 2008). This study extended water analysis from the natural environment of Alaska to the rural and urban communities of California. The geographical sampling region for rural and urban ponds were the eastern Pacific flyway for migratory birds in California. Surface water samples were collected from artificial recirculating ponds in rural and urban areas. There are 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l).

Primary data were collected using commercially available influenza A viral M gene RT-PCR detection kits suitable for water analysis, influenza A virus viability through viral culture assay using Madin Darby canine kidney cell lines, and gene sequencing for influenza A H5N1 viral subtypes following WHO recommended guidelines for H5N1 primer sequences from a commercial reference laboratory.

The social change implications of this dissertation can be recognized at the national and international levels, to the population level, and to the individual level. At the national and international level, I attempted to contribute geospatial analysis and spatial-temporal data for influenza A surveillance. H5N1 influenza virus surveillance may detect highly pathogenic avian influenza virus H5N1 presence along the eastern Pacific flyway. The detection of highly pathogenic avian influenza virus H5N1 may initiate biosecurity measures to protect poultry industries in the United States and Brazil. The United States and Brazil are the world's largest exporters of poultry (Butler & Ruttimann, 2006). Additionally, I attempted contribute new H5N1 viral genome

sequences to the current influenza A virus H5N1 database. An increased database may provide tools for avian influenza A virus H5N1 vaccine development.

Vaccine developments were positive social change at the population level. Soda, et al. (2008) suggested the applicability of a virus strain library for vaccine preparation. The results of this dissertation may contribute to the current virus strain library at GenBank. Vaccine development may have may positive social change for American Indian/Alaskan Natives.

The Centers for Disease Control and Prevention (2009) reported American Indian/Alaskan Natives had H1N1 mortality rates four times higher than persons in all other racial/ethnic populations combined. Poverty and poor living conditions may predispose American Indian/Alaskan Natives to influenza complications (Centers for Disease Control and Prevention, 2009). Chakraborty, Papageorgiou, and Sebastián (2008) suggested the disproportionate share of a developing country disease burden is due to infectious diseases. Applying this hypothesis to American Indian/Alaskan Natives, whose H1N1 mortality rates were four times higher than persons in all other racial/ethnic populations combined, may explain the poverty and poor living conditions that may predispose this population to influenza complications. At the individual level the findings of this dissertation may bring about greater awareness of influenza A virus infection.

Shive and Kanekar (2011) suggested the principles of the Health Belief Model can provide explanation why many people, including high risk individuals, do not receive influenza vaccination. This dissertation study may bring greater awareness to the individual, and thus, greater perceived susceptibility to influenza A virus infection.

Therefore, the findings of this dissertation study may result in improvements in vaccinations for American Indian/Alaskan Natives resulting in positive social change.

In this dissertation study, I analyzed samples from artificial recirculating water ponds. Artificial recirculating water ponds are often a decorative feature in parks and golf courses. The decorative feature is often constructed to attract people. Also, these decorative features may attract both domestic and migratory waterfowl (Webster et al., 1992). Migratory waterfowl likely will introduce influenza A viruses to the artificial recirculating water pond enivronment from viral shedding in feces. If the artificial recirculating water pond is constructed with an aeration fountain, mist and droplet formation may occur. As is well established, influenza viruses are transmitted between individuals through droplets (e.g., coughing, sneezing). An individual who comes in contact with the droplets from the contaminated artificial recirculating water pond may be inoculated with influenza A virus. Therefore, the findings of this dissertation study may bring greater awareness at the individual level, and thus, increase the perceived susceptibility to influenza A virus infection. As implied by Shive and Kanekar (2011), perceived susceptibility to influenza A virus infection will have a direct correlation to influenza vaccination.

Summary

This introduction included a moderately detailed presentation of my dissertation study. Main points of this chapter include a brief discussion of the research literature including, gaps in the knowledge and gaps in the current literature. These discussions were followed by discussion of the problem statement, necessity of the problem and the

relevance of the problem. Following brief discussion of the problem, are discussions of the research questions, theoretical framework, and the relationship of the theoretical framework to the study approach and research questions. Lastly, the nature of the study including definitions, assumptions, scope and delimitations, limitations, and significance were discussed. Following this brief introduction of my dissertation study is the literature review.

The literature review of this manuscript is organized into fourteen sections. The fourteen sections are arranged to present influenza topics in an hourglass format beginning with the broad historical background of influenza surveillance, through pandemic influenza outbreaks, to government responses. Following the historical review are the less broad sections of the hypothesis of the origin of novel influenza viruses, Mixing Vessel Theory to explain the emergence of novel influenza viruses, migratory waterfowl as vectors for global distribution of influenza viruses, and wetland breeding grounds as hotspots for influenza virus detection. After discussion of influenza in the aquatic environment, the topic will narrow to water analysis, the molecular structure of the influenza viron, and laboratory assays. Subsequent to the discussion of laboratory assays, the topic will broaden to present current influenza surveillance and risk assessment tools, and social change implications. Preceding the review of the literature is the theoretical foundation of this dissertation study, the conceptual framework, and the epidemiological triangle as it applies to pandemic influenza disease.

Chapter 2: Literature Review

The “Spanish” influenza H1N1 pandemic of 1918–1919, which caused approximately 50 million deaths worldwide, remains an ominous warning to public health (Taubenberger & Morens, 2006). Since then, new subtypes of human influenza A viruses have been detected at various times: in 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, the H3N2 subtype (Hong Kong virus) appeared; and in 1977, the H1N1 virus reappeared (Webster et al., 1992). Influenza disease emergence data are collected year-round, but economic strain on global public health to prevent and treat human influenza outbreaks is enormous. Therefore, it is imperative to identify potential sources of the virus to help minimize outbreak occurrence.

Numerous research studies have indicated migratory waterfowl are a source point for influenza viruses (Austin & Hinshaw, 1984; Ferro et al., 2010; Gaidet et al., 2010; Webster et al., 1992). The literature review revealed five key points. First, the long-range migratory patterns of waterfowl could explain the global dispersion of influenza viruses. Second, select migratory waterfowl species potentially are the natural reservoir for influenza viruses. Third, viral antigenic shift occurs by a pre- or post transmission mechanism from host species to recipient species. Fourth, the transmission of influenza virus occurs between waterfowl through a water-borne fecal to oral mechanism. Fifth, waterfowl breeding grounds in the Alaskan watershed, Siberian lakes, and other migratory waterfowl breeding habitats are potential “hot-zones” for water-borne transmission of influenza virus. Even though many investigated the persistence of influenza viruses in aquatic habitats, no known research has analyzed the proportion and

persistence of influenza viruses in artificial suburban neighborhood water ponds. The aim of this dissertation study was to investigate the proportion of influenza A viruses in recirculating artificial ponds in rural and urban geographical locations. The proportion of influenza A virus in rural and urban geographical locations may be dependent on ecological and contextual aspects of influenza viruses.

There are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. Gaps in understanding the role of the physical and biogeochemical environment as an integral part of the influenza A viral transmission also exist (Lang et al., 2008). More importantly, gaps in knowledge about the burden of influenza A virus in rural and urban community settings remain present. Researchers have expected IRAT to prompt additional studies to address these key gaps in the knowledge (Trock et al., 2012).

The problem has been public health scientists have been battling emerging human influenza diseases with tactile and reactionary methods because of a lack of knowledge and data at the human-animal interface. The purpose of this baseline study of the proportion of influenza A virus in urban and rural community settings was to provide knowledge and biological data of significant interest at the human-animal interface. This biological data may be of significant interest for the development of IRAT.

International Level

International scientific collaboration suggested pandemic influenza viruses may arise months prior to their emergence in humans, providing a window for the

identification and implementation of preparedness and response activities before a pandemic occurs (World Health Organization, 2011). The 2009 H1N1 outbreak in Central and South America initiated emergency response and leadership by the World Health Organization and the Pan American Health Organization. The leadership efforts by these two organizations aided many municipalities and provinces to formulate policies to respond to the influenza A virus H1N1 outbreak (Stern, Koreck, & Markel, 2011).

National and State Level

A review of state-level pandemic influenza preparedness plans for the United States by Thomas and Young (2011) revealed most state plans were incomplete with many lacking ethics preparedness. Ethics preparedness and planning include the allocation of vaccine and antiviral medications, legal authority, and quarantine. California is a state with numerous ethics preparedness recommendations but without implementation of these recommendations. The primary barrier for lack of ethics preparedness implementation is a "lack of appreciation for, or priority given to, public health ethics among those receiving the recommendations" (Thomas & Young, 2011, p. 2081).

Populational Level

At the population level, personal risk perception and health communication regarding influenza infections has been an emerging and challenging area for public health professionals (Shive & Kanekar, 2011). Shive and Kanekar (2011) identified vulnerable populations such as rural residents where improvements in the understanding between animal disease, human behavior, and the risk of influenza infection needs

improvement. Many state ethics preparedness plans are mostly incomplete or without implementation at the rural and/or urban community level. A purpose of this dissertation was to bring about greater awareness of the relationship the human-animal interface and preparedness at the rural and urban community level. Thus, the information about ethics preparedness was relevant to this study.

Human–Animal Interface

Artificial water ponds in rural and urban communities are sites of the human-animal interface. Webster et al. (1992) described this human-animal interface with the example of domestic ducks in community ponds attracting migratory waterfowl. The migratory waterfowl introduce the influenza virus to that community's water pond from fecal contamination. The contaminated community water pond now becomes a potential source of influenza virus to both humans and animals.

Franklin et al. (2011) proposed aquatic systems may serve as reservoirs and sources of infection for both wild birds and mammals. Further, Franklin et al. suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals. An investigation of the burden of influenza A virus in artificial rural and urban ponds may identify aquatic systems that serve as reservoirs for influenza A virus as described by Franklin et al.. An investigation of the human-animal interface in these aquatic habits may also bring about significant social change.

Social Change

Globally, pandemic 2009 influenza A (H1N1) virus infection has been found to cause a disproportionately higher rate of hospitalization and death among indigenous populations from Australia, Canada, and New Zealand (Centers for Disease Control and Prevention, 2009). Indigenous populations in the United States have also shown a disproportionately higher rate of hospitalization and death from pandemic 2009 influenza A (H1N1) virus infection. The Centers for Disease Control and Prevention (2009) reported American Indian/Alaskan Natives had H1N1 mortality rates four times higher than persons in all other racial/ethnic populations combined.

The increased mortality among the indigenous people of the United States may be due to social injustice. These social injustice conditions may include high prevalence of chronic health conditions (e.g., diabetes and asthma) among American Indian/Alaskan Natives that predisposes them to influenza complications, poverty (e.g., poor living conditions), and delayed access to care (Centers for Disease Control and Prevention, 2009). The Centers for Disease Control and Prevention (2009) recommended increased efforts to increase awareness among American Indian/Alaskan Natives and their health-care providers of the potential severity of influenza and current recommendations regarding the timely use of antiviral medications. Investigation into the human-animal interface in rural communities may lead to increased awareness among American Indian/Alaskan Natives and their health-care providers of the potential severity of influenza A viruses. Thus, the results of this dissertation study may bring about positive social change among the American Indian/Alaskan Native people of the United States.

Significance

Presently, no known research is underway to investigate and compare the proportion of influenza A virus in artificial rural and urban ponds. A review of current literature did not identify any other studies that attempted to collect influenza A virus data from artificial water ponds in rural or urban communities. Several researchers investigated the presence or persistence of influenza viruses to natural waterfowl wetlands (Austin & Hinshaw,1984; Ferro, et al., 2010; Lang et al., 2008). The purpose of this quantitative study is to extend the previous environmental virology research of influenza in natural waterfowl wetlands to rural and urban communities. The discovery of the proportion of influenza A virus in rural and urban communities will establish baseline measures for future monitoring and impact studies in these settings. These baseline measures may initiate surveillance data for IRAT programs.

Even though the IRAT is not designed to predict the next pandemic, IRAT is expected to identify gaps in the knowledge, prompt further research, assist with clear documentation of the thought process, and focus risk management efforts (Trock et al., 2012). The development of baseline measures of the environmental burden of influenza A virus in communities will inaugurate new data collection for IRAT.

Current Literature and the Relevance of the Problem

Seasonal influenza virus outbreaks cause 250,000 to 500,000 deaths annually (Keeler, 2011). The review of the literature identified many studies and publications seeking to reduce the morbidity and mortality resulting from influenza virus outbreaks. One area of significant interest is the human-animal interface. The human-animal

interface is recognized as a complex but critical juncture to which new paradigms are emerging (World Health Organization, 2011). Aquatic virus reservoirs is a human-animal interface. Franklin et al. (2011) suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals. The review of the literature identified several studies that establish the relevance of the problem. The problem is there are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses.

Austin and Hinshaw (1984) investigated feral duck species as a source of transmission of influenza A virus and paramyxoviruses. The authors of this study suggested surveillance of healthy ducks and the aquatic environment they frequent may be of significant interest to monitoring and controlling influenza A viruses (Austin & Hinshaw, 1984). Austin and Hinshaw and others have identified influenza viral subtypes in various waterfowl breeds (Ferro, et al., 2010; Mehrabanpour, et al., 2012). These studies support the hypothesis that migratory waterfowl play a role in the natural influenza virus reservoir(s) and dispersal. Similar studies have been conducted in the United States.

Ferro et al. (2010) investigated the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas Coast. Other researchers have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen et al., 2010; Webster et al.,

1992). VanDalen et al. (2010) were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Thus, it is possible that influenza viruses can be transfered between organisms via open water-sources as wetlands, lakes, and ponds. Other researchers have investigated if influenza A viruses can be detected in these contaminated aquatic habitats.

Lang, et al. (2008) collected and studied sediment samples from three ponds in the Creamer's Field Migratory Waterfowl Refuge, Alaska, a location used by a wide variety of migratory waterfowl. The sediment samples were collected using a time-series approach and analyzed for influenza A virus RNA using reverse transcription-polymerase chain reaction (RT-PCR) methodologies (Lang et al., 2008). Zhang et al. (2006) collected and studied samples of ice or water from three northeastern Siberian lakes in the Koluma River region. The collected samples were analyzed for the presence of influenza A virus using reverse transcription-polymerase chain reaction (RT-PCR) methodologies (Zhang et al., 2006). Other researchers have studied influenza A viruses in surface water samples to investigate the perpetuation between waterfowl and water-contamination (Hinshaw et al., 1980; Ito et al., 1995), while others have investigated infectivity and transmission of influenza A viruses in the aquatic environment (Brown et al., 2009; Halvorson et al., 1985).

In the longitudinal study by Hinshaw et al. (1980), researchers collected and analyzed samples from waterfowl, unconcentrated lake water, and feces from lake shores near Vermillion, Alberta, Canada. These samples were used to isolate influenza viruses and to investigate whether influenza viruses continually circulate or whether the same or

different strains are present from year to year (Hinshaw et al., 1980). Hinshaw et al. hypothesized the longitudinal study of feral ducks would provide data on the size of the gene pool of influenza A viruses in nature and the number of viruses antigenically related to human strains present in avian species.

Ito et al. (1995) investigated influenza virus isolates from collected from the fecal samples of dabbling ducks and also from lake water used by migratory waterfowl. The results of this investigation showed the viruses remained viable in the lake water after most ducks left for migration South (Ito et al., 1995). Brown et al. (2009) asserted the pH, temperature, and salinity-at levels normally encountered in nature-can impact the ablity of avian influenza A viruses to remain viable in water. Halvorson et al. (1985) investigated the theory of water-borne transmission of avian influenza A viruses among wild waterfowl and domestic fowl. However, the research only identified certain duck-derived isolates are capable of infecting domestic turkeys (Halvorson et al., 1985). Further investigation into the molecular structure of the influenza viron were published by several researchers.

The work by Poulson (2011) and Taubenberger and Kash (2010) is a current detailed understanding of the molecular structure of influenza A virus. These eight single-stranded RNA gene sequences are: PB2, PB1, PA, HA, NP, NA, M, and NS (Taubenberger & Kash, 2010). The methodologies to investigate and understand the molecular structure of influenza A virus include: virus detection by RT-PCR (Poddar et al., 2002), virus isolation and characterization by allantoic cavities of embryonic eggs (Fouchier, et al., 2005), infectivity studies using MDCK cell line (Brown et al., 2009),

nucleotide sequencing of the RNA genome (Yamamoto, et al., 2011), and phylogenic analysis using BLAST available from GenBank (Fouchier, et al., 2005).

Zhang et al. (2011) asserted the Dongting Lake wetland as an important habitat and over-wintering area for East Asian migratory birds. From the 95 water samples collected by Zhang et al., an H10N8 influenza A virus was isolated, whole genome genetic sequenced, and pathogenicity investigated in mice and specific pathogen free (SPF) White Leghorn Chickens. The study by Zhang et al. further supports environmental water sampling approach for influenza risk assessment. The review of the literature identified many studies and publications supporting the material and methods of this dissertation study.

The Zhang et al. (2011) study is support for 200-ml water sample collection in areas near the habitat of migratory birds and storage of the samples at -80°C until assayed. Tønnessen, et al. (2013) investigated Mixing Vessel Theory, sampling along migratory flyways for influenza viruses, genome sequencing, and similarity analysis of the findings in this dissertation research. Evers et al. (2007) investigated the commercial preservative RNAlater (Qiagen) against the current method of cryo-freezing, and ethanol preservatives for influenza A virus samples. The purpose of this investigation was to determine if using the commercial preservative RNAlater would result in improved RT-PCR amplification over the current sample preservation methods of cryo-freezing or ethanol fixation (Evers, Slemons, & Taubenberger, 2007).

The review of the literature identified many studies and publications seeking to reduce the morbidity and mortality resulting from influenza virus outbreaks. The

researchers and scientists of these studies sought to investgate gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. The literature search strategy of this dissertation identified studies that establish the relevance of the problem and support the investigation of the burden of influenza A virus in rural and urban ponds.

Literature Search Strategy

A literature search was conducted using (i) relevant textbooks; (ii) electronic bibliographic databases, namely CINAHL, Medline, Academic Search Premier, JSTOR, Science Direct, Wiley Interscience Journals, Science, and Nature; (iii) reference lists of articles published in relevant journals; and (iv) Internet websites for the Centers for Disease Control and Prevention, and the World Health Organization. English and non-English language articles and books published between 1844 and 2013 were included in this study. The search for relevant textbooks was based on more general terms such as influenza, pandemic, viral biology, and molecular biology. The following key words were used for the electronic databases: influenza, pandemic, viral biology, molecular biology, antigenic shift and antigenic drift, influenza virus RT-PCR, migratory waterfowl, and influenza phylogenic analysis. Several hundred references were found including textbooks or reports and articles. From the findings, 167 references were selected including textbooks (n = 6), reports (n = 35), articles (n = 124), and other (n = 2).

The literature review of this manuscript is organized into fourteen sections. The fourteen sections are arranged to present influenza topics in an hourglass format

beginning with the broad historical background of influenza surveillance, through pandemic influenza outbreaks, to government responses. Following the historical review were the less broad sections of the hypothesis of the origin of novel influenza viruses, Mixing Vessel Theory to explain the emergence of novel influenza viruses, migratory waterfowl as vectors for global distribution of influenza viruses, and wetland breeding grounds as hotspots for influenza virus detection. After discussion of influenza in the aquatic environment, the topic will narrow to water analysis, the molecular structure of the influenza viron, and laboratory assays. Subsequent to the discussion of laboratory assays, the topic will broaden to present current influenza surveillance and risk assessment tools, and social change implications. Preceding the review of the literature is the theoretical foundation of this dissertation study, the conceptual framework, and the epidemiological triangle as it applies to pandemic influenza disease.

Theoretical Foundation

The theoretical framework for this study were based on the hypotheses proposed by Webster et al. (1992) and others that (i) migratory waterfowl are the natural reserve of influenza viruses, and (ii) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. Adding to the theoretical framework were Scholtissek et al. (1985) Mixing Vessel Theory. Mixing Vessel Theory posits the viral adaptation from host animal to human infectivity occurs in an intermediate host animal such as a swine or waterfowl (Ma et al., 2009).

The literature suggests novel influenza viruses originate in the Southern China region where migratory waterfowl become infected (Webster et al., 1992). Infected

asymptomatic migratory waterfowl transport the novel influenza viruses in their intestinal tracts to the wetland breeding grounds of Alaska and Siberia (Gaidet, et al., 2010). The water-borne transmission of the influenza viruses to new hosts (migratory waterfowl from Northern and Southern American regions) occurs at these Alaskan and Siberian wetland breeding grounds (Lang et al., 2008). The new host(s) may act as a mixing vessel for influenza viruses. This may result in an antigenic shift or antigenic drift of influenza viruses resulting in novel strains (Ma et al., 2009). Infected asymptomatic migratory waterfowl may then transport these influenza virus strains along the eastern Pacific flyway for migratory birds (Carver, 2003). The eastern Pacific flyway for migratory birds extends from the southern regions of South America to Alaska. Thus, novel influenza viruses originating in the southern China region can be transported to the North, Central, and South American regions.

Underlining the theoretical framework for this study were Pasteur's (1857-1858) Germ Theory and Koch's (1890) Postulates as adapted for viruses. Pasteur's Germ Theory identifies germs of microscopic organisms as the cause of disease, and the theory of spontaneous generation is chimerical (Pasteur et al., 1878). Koch's Postulates as adapted for viruses implies that certain conditions-not all of Koch's Postulates-have to be met before a specific relation of a virus to a disease is established. The conditions are: (i) a specific virus must be found associated with a disease with a degree of regularity, and (ii) the virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation (Rivers, 1937).

Conceptual Framework

Recent work by Taubenberger and Kash (2010) is an analysis of the current understanding of the molecular mechanisms of influenza viruses to human adaptation. The research reviewed by Taubenberger and Kash focused on three subject areas of influenza: (i) molecular structure of influenza virus, (ii) the evolutionary dynamics of antigenic shift and drift, and (iii) phylogenic analysis of isolated viruses in animals and humans. The fundamental question of how avian influenza viruses switch host remains unanswered. Additionally, it is poorly understood how the viral genetic changes effect human adaptation, human-to-human transmissibility, and pathogenesis (Taubenberger & Kash, 2010). To further understand the characterization of influenza viruses, some researchers have approached the topic of avian influenza through the lens of the epidemiological triangle.

Epidemiological Triangle

The epidemiological triangle has three vertices: host, agent, and environment. The host is the organism harboring the disease. For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) (Franklin et al., 2011). The agents are the influenza A viruses. The environments are the natural and artificial habitats where the hosts are found. The review of the literature identified many articles where one or more of the verticles of influenza infection was investigated.

Perez-Ramirez et al. (2011) studied avian influenza in wild birds in Europe, Asia, and Africa. This article includes detailed information about host species, the presence of low-pathogenic and high-pathogenic avian influenza, origins of the influenza viruses,

mechanisms of spread, and geographical areas where the infected birds have been detected (Perez-Ramirez et al., 2011). Webster et al. (1992) included detailed research about reservoirs of influenza A viruses in nature, the structure and function of the influenza virus viron, and mechanisms for the perpetuation of influenza viruses in avian species. Avian species are the reservoir host for influenza viruses.

Host. The burden of influenza A viruses in nature is widespread (Lebarbenchon & Stallknecht, 2011). Lebarbenchon and Stallknecht (2011) stated avian influenza virus has been isolated in more than 105 different bird species worldwide where wild waterfowl (ducks, geese, and swans) are recognized to be the natural host. Franklin et al. (2011) supported the hypothesis that waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) are the animal reservoirs for avian influenza virus. Additionally, Franklin et al. proposed the natural transmission cycle of avian influenza virus as: first, viral replication in the gastrointestinal tract of an aquatic bird; second, high concentrations of virus shed into the aquatic environment through feces; third, sustained Avian influenza virus viability in aquatic environments; and fourth, subsequent avian influenza virus infection of other animals in the same aquatic environment. This natural transmission cycle of avian influenza virus proposed by of the Franklin et al. is known as water-borne transmission.

The hypothesis of water-borne transmission of influenza virus has been the foundation for several studies investigating the characterization of influenza viruses in waterfowl (Ferro, et al., 2010), in surface water (Lebarbenchon, et al., 2011), and in sediment samples (Lang et al., 2008). Austin and Hinshaw (1984) investigated feral duck

species as a source of transmission of influenza A virus and paramyxoviruses. The researchers collected swab samples from tracheal and cloacae from different species of feral ducks and were able to identify several viral strains of influenza A virus (Austin & Hinshaw, 1984). The alterations of influenza viral strains occur due to antigenic drift due to point mutations and antigenic shift caused by genetic reassortment. Austin and Hinshaw asserted that it was possible these alterations occurred in the intestinal tract of ducks. The Austin and Hinshaw study is support for the hypothesis that migratory ducks are a source point for influenza viruses. This article also includes data that suggests asymptomatic and healthy ducks can host a variety of influenza strains.

Gaidet et al. (2010) focused on the long-range migratory patterns of waterfowl to explain the global dispersion of highly pathogenic avian influenza H5N1 virus (HPAI H5N1). Satellite telemetry was used to study the movements of migratory waterfowl over Asia, Europe, and Africa. The authors concluded, on average, a migratory bird would only have 5-15 days per year of infection to disperse HPAI H5N1 (Gaidet, et al., 2010). Thus, intercontinental virus dispersion requires relay transmission between series of successfully infected migratory waterfowl. Additionally, only asymptomatic birds could successfully disperse HPAI H5N1 (Gaidet, et al., 2010). This study supports the theory the seasonality of influenza outbreaks can be explained, in part, by movements of migratory birds. However, population studies of migratory birds are not a reliable influenza disease surveillance tool.

Hierarchical modeling can be used to estimate bird populations. Link and Sauer (2002) focused on hierarchical modeling for estimating Cerulean Warblers populations

using North American Bird Breeding Survey data. The work by Link and Sauer is critical of the validity of North American Bird Breeding Survey data. The authors asserted North American Bird Breeding Survey data is not well suited for monitoring the Cerulean Warblers (Link & Sauer, 2002). Reasoning for this critical judgment is the flight patterns of the Cerulean Warblers are not compatible with the observation methods of North American Bird Breeding Survey. This article provides opinion about the application and validity of the population trends of the North American Bird Breeding Survey data. Additionally, this article rules-out the association of migratory bird population data to seasonal influenza outbreaks. However, the studies of migratory waterfowl as a host is still of great importance in influenza A virus surveillance.

Mehrabanpour, Rahimian, Shirazinezhad, Moein, and Shayanfar (2012) focused on cloacal swabs and samples of bird droppings from the wetland regions of Boushehr, Iran. Samples were analyzed for influenza viral strains using RT-PCR methodologies. These studies and others have identified influenza viral subtypes in various waterfowl breeds (Ferro, et al., 2010; Mehrabanpour, et al., 2012). These studies support the hypothesis that migratory waterfowl play a role in the natural influenza virus reservoir(s) and dispersal. Similar studies have been conducted in the United States.

Ferro et al. (2010) focused on the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas Coast. Cloacal swabs were analyzed for influenza A viruses using RT-PCR technique. Over a three year period, the researchers were able to collect samples from 5,363 birds. Data analysis showed a prominence of specific sub-strains of influenza viruses and also identified Blue-winged

Teals and Green-winged Teals as carriers of several influenza viral subtypes (Ferro, et al., 2010). These studies provide additional evidence that influenza viruses can be identified among health migratory waterfowl. A different approach to isolating influenza viruses was conducted in Pennsylvania.

Henzler et al. (2003) investigated the avian influenza H7N2 outbreak in Pennsylvannia during the years 1996-1998. In this study, the researchers captured and culled wild-mice from 10 poultry premises where avian influenza H7N2 had been identified. The authors reported that all of the wild-caught mice intestinal tissue and lung pool specimens were negative for influenza virus even though poultry on the premises were positive for avian influenza (Henzler, et al., 2003). The research model investigated by Henzler et al. rules-out wild-mice as host, and as a surveillance tool for influenza viruses. Other researchers have investigated other organisms living within the influenza A virus environment.

Faust, Stallknecht, Swayne, and Brown (2009) focused on the ability of filter-feeeding bivalves to remove influenza virus from contaminated water. The researchers were able to demonstrate that filter-feeding bivalves can remove influenza virus from contaminated water, however, the findings of the research suggest that avian influenza virus is inactivated or sequestered in clam tissue after filtration (Faust, Stallknecht, Swayne, & Brown, 2009). The research model investigated by Faust et al. rules-out filter-feeeding bivalves as host, and as a surveillance tool for influenza A viruses. These researchers investigated potential host species for influenza A viruses. Others have focused investigation on the agent, influenza A virus, itself.

Agent. Moattari et al. (2010) studied the phylogenic pathways of antigenic variations of human influenza viruses. The analysis of 300 pharyngeal swab samples from influenza patients identified several similarities among different influenza viral subtypes. The purpose of these studies was to compare the effectiveness of a common vaccination formula to influenza virus (Moattari, et al., 2010). Antigenic shift and phylogenic analysis of the samples provide evidence and support that RNA mutations in the influenza virus genome can result in novel outbreak strains. A novel outbreak strain from a common virus supports the hypothesis that mutagenic change occurs within a single organism, namely a migratory waterfowl.

Saitou and Nei (1986) posit an explanation to the process of viral evolution for influenza A viruses. Additionally, this manuscript provides operational definitions of the terms: phylogenic tree, polymorphism, mutation rate, and nucleotide substitution (Saitou & Nei, 1986). Recent studies provide more detailed description of the molecular mutations and evolution of influenza viruses (Taubenberger & Morens, 2006). These early studies relate the theory of viral evolution to influenza viruses.

Environment. The literature suggests novel influenza viruses originate in the Southern China region (Webster et al., 1992). Migratory waterfowl become infected with the novel influenza viruses in this region. Asymptomatic migratory waterfowl transport the influenza viruses in their intestinal tracts to the wetland breeding grounds of Alaska and Siberia (Gaidet, et al., 2010). The water-borne transmission of the influenza viruses to new hosts (migratory waterfowl from Northern and Southern American regions) occurs at these Alaskan and Siberian wetland breeding grounds (Lang et al., 2008). The

new host(s) may act as a mixing vessel for influenza viruses. This may cause an antigenic shift or antigenic drift of influenza viruses resulting in novel strains (Ma et al., 2009). Infected asymptomatic migratory waterfowl from Northern and Southern American regions may then transport these influenza virus strains along the eastern Pacific flyway for migratory birds (Carver, 2003). The eastern Pacific flyway for migratory birds extends from the southern regions of South America to Alaska. Others have investigated the transmission to new hosts in the live bird market environments of southern China.

Martin, et al. (2011) investigated the live-bird markets of Southern China as a potential environment for the transmission of avian influenza viruses. The researchers in this mixed-methods study interviewed live poultry traders to flesh out incoming and outgoing trading activities. Additionally, the researchers collected a total of 7050 cloacal and tracheal swabs, 2415 environmental, and 610 water samples from 30 market nodes to investigate the burden of highly pathogenic avian influenza H5N1 (Martin, et al., 2011). From the results of the Martin, et al. (2011) study, the authors concluded that risk-based surveillance of the live bird markets of Southern China positively supplements China's effort and commitment to highly pathogenic avian influenza H5N1 control. Literature by others have identified human-to-human transmission as an opportunity to control influenza A virus transmission.

Animal-to-human transmission is considered "primary" transmission and human-to-human transmission of zoonotic diseases is considered "secondary" transmission (Lloyd-Smith, et al., 2009). McClure et al. (2011) recognized personal hygiene and vaccination as the most important and effective methods for controlling for spread of

influenza viruses. Mao and Yang (2012) posit that human networks, infectious diseases, and human preventive behavior are intrinsically inter-related. Personal hygiene is an extension of human preventive behavior for controlling the spread of influenza viruses in the modern environment.

History of Influenza Surveillance

In 1947, at the 4th International Congress for Microbiology held in Copenhagen, a group of virologist forwarded a recommendation to the Interim Commission of the World Health Organization an international program be initiated for influenza surveillance (Hampson, 1997). The World Health Organization Global Influenza Programme was established later that year (Fleming, van der Velden, & Paget, 2003). The 1947 influenza surveillance control programme is the oldest disease control program at the World Health Organization (World Health Organization, 2005). The World Health Organization (2005) stated the creation of the 1947 influenza surveillance control programme resulted from two concerns: first, the inevitable recurrence, at unpredictable intervals, of highly disruptive pandemics; and second, the significant health and economic impact of seasonal epidemics, which occur nearly every year (p. 34). Hampson (1997) opinioned the objectives of the 1947 influenza surveillance control programme as: to gain an understanding of the epidemiology of influenza, and to promptly isolate influenza viruses from new outbreaks and distribute them for vaccine production (p. S8). Within four years of creation, the 1947 influenza surveillance control programme developed into a network of 60 laboratories across 40 countries (World Health Organization, 2005).

The influenza surveillance control programme became the WHO Global Influenza Surveillance Network consisting of 113 national influenza centers located in 84 countries (World Health Organization, 2005). Following the adoption by the World Health Organization of the Pandemic Influenza Preparedness Framework in May 2011, the Global Influenza Surveillance Network was changed to Global Influenza Surveillance and Response System. The Centers for Disease Control and Prevention and the European Influenza Surveillance Scheme (EISS) collaborate with the WHO Global Influenza Surveillance and Response System to monitor the evolution of influenza viruses and provides recommendations in various disciplines including laboratory diagnostics, vaccines, antiviral susceptibility and risk assessment (World Health Organization, 2013). The CDC developed its own surveillance control program during the same period. Unlike the WHO surveillance programme which originated from public health concern about influenza disease, the CDC surveillance program originated from public health concern about malaria disease.

The Communicable Disease Center was established July 1, 1947 for the purpose of continuance of the Malaria Control in War Areas organization into peacetime (Andrews, 1946). Andrews (1946) described the concern of the U.S. Public Health Service that servicemen returning from Malaria endemic war areas may act as a reservoir and a mode of transmission of Malaria viruses (Andrews, 1946). Andrews further depicted the early organization of the Communicable Disease Center as a result from revisons of the Malaria Control in War Areas organization. The early administrative and technical divisions of the Communicable Disease Center include: (i) Epidemiology, (ii)

Laboratory, (iii) Engineering, (iv) Entomology, (v) Technical Development, (vi) training, (vii) Production, and (viii) Library and Reports (Andrews, 1946). Over the next decade, the focus changed from malaria to influenza.

On June 18, 1957 a memorandum from William A. Neill, M.D., Chief, Epidemic Intelligence Service, and Alexander D. Langmuir, M.D., Chief, Epidemiology Branch, with the subject: *Alert to Possible Influenza Outbreaks* was addressed to all Epidemic Intelligence Service Officiers and other professional personnel at the Epidemiology Branch (Neill & Langmuir, 1957). Neill and Langmuir (1957) wrote the purpose of the memorandum was to bring awareness of the 1957 Far East influenza outbreak. The following month, on July 9, 1957 the *CDC Influenza Report No.1* was produced.

The emphasis of the *CDC Influenza Report No.1* was to provide summary information regarding outbreaks and cases of confirmed infections of the Far East strain of influenza virus, outbreaks of influenza-like illness with specimens under laboratory study, and outbreaks found not to be influenza or epidemics of influenza-like illness with no specimen available (Communicable Disease Center, 1957). The title *CDC Influenza Report* was changed to *CDC Influenza Surveillance Report* in Fall of 1957. On October 1, 1957 the *CDC Influenza Surveillance Report No. 19* was released. The emphasis of the *CDC Influenza Surveillance Report No. 19* was to provide a summary of the continued nationwide spread of the Asian Strain Influenza 1957 (Communicable Disease Center, 1957).

The title *CDC Influenza Surveillance Report* was changed to *Influenza Surveillance Report* in early of 1963. On February 15, 1963 the Influenza Surveillance

Report No. 74 was released. The emphasis of *Influenza Surveillance Report No. 74* was to provide a state-by-state summary of outbreaks and cases of influenza and influenza-like illness (Communicable Disease Center, 1963). The title *Influenza Surveillance Report* was changed to *Influenza-Respiratory Disease Report* in mid-1965. The new name reflected the intended expansion of content in future *Influenza Surveillance Summaries* resulting as the epidemiology of multitudinous respiratory viruses was better documented (Communicable Disease Center, 1965). Currently, the Epidemiology and Prevention Branch in the Influenza Division at the Centers for Disease Control and Prevention collect, compiles, and analyzes data on influenza activity throughout the United States and produces *FluView*, a weekly influenza surveillance report (Centers for Disease Control and Prevention, 2011). The European Centre for Disease Prevention and Control has a similar influenza surveillance program as the CDC named European Influenza Surveillance Network.

The European Influenza Surveillance Network collects influenza surveillance data from all European Union countries, Iceland and Norway (European Centre for Disease Prevention and Control, 2013). The origin European Influenza Surveillance Network can be traced back to sentinel practice-based surveillance for influenza-like illness in the United Kingdom. Fleming, van der Velden, and Paget (2003) provided a historical account of early 1970s surveillance schemes in the Netherlands, Scotland, and Portugal based on the practice-based surveillance of the United Kingdom. During the 1980s, sentinel surveillance networks were established in France, Belgium, and Spain (Fleming

et al., 2003). Fleming et al. (2003) further detailed the 1989 European Commission funding of the Eurosentinel Project.

A primary goal of the Eurosentinel Project was to unify the influenza surveillance networks of each European country (Fleming et al., 2003). In the ensuring years, the Eurosentinel Project became the ENS-CARE Influenza Early Warning Scheme, followed by the European Influenza Surveillance Scheme (European Centre for Disease Prevention and Control, 2013). Currently, the European Centre for Disease Prevention and Control (2013) issues the *Weekly Influenza Surveillance Overview* which is a surveillance report based on the data collected by sentinel physicians and data collected and processed by national co-ordination centers among the member states.

Modern influenza surveillance began with the World Health Organization Global Influenza Programme in 1947. Since then, many nations and international collaborations have evolved due to advancements in research and science. Throughout this evolution, the overarching purpose of influenza surveillance has remained the same: to reduce the global burden of influenza viruses on the human population. The understanding of the history of influenza surveillance aids in aligning the research of this dissertation. Historical study of influenza outbreaks further focuses the research of this dissertation.

Pandemic Flu

Influenza viral infections appear with regular annual frequency that the phenomenon is common to refer to period as ‘flu season” or “seasonal flu”. However, pandemic influenza events have not been regular or predictable. Taubenberger and Morens (2006) identify and provide a brief synopsis of 13 pandemic events between 1510

and 1978. Wali and Music (2011) provided a summary of public health outcomes of nine U.S. influenza epidemics that occurred between the period 1972 – 1973 to 1991 – 1992. Keeler (2011) provided a brief history of influenza pandemics from the time of discovery of the influenza virus through the last decade. Further, the work by Keeler notably identified the sharp increase in public awareness of influenza and the potential human health threat of the virus. One of the greatest human helath threats was the pandemic Spanish flu of 1918.

1918-1919 (Spanish Flu)

A review of the literature describes the pandemic Spanish flu of 1918 as one of the deadliest disease outbreaks in history (Keeler, 2011; Morens & Fauci, 2007). Taubenberger and Morens (2006) portrayed the pandemic Spanish flu of 1918 as "…remaining as an ominous warning to public health" (p. 15). Patterson and Pyle (1983) pictured the pandemic Spanish flu of 1918 as "…one of the most pervasive and devastating biological disasters since the bubonic plague of the fourteenth century" (p. 1299). A review of the literature further provided past and current knowledge of the Spanish flu of 1918 pandemic.

The current knowledge of the pandemic Spanish flu of 1918 stimulates additional discussion and guides researchers to gaps in the knowledge. Oxford (2000) contended there are several important and practical questions to ask about the pandemic Spanish flu of 1918: (i) Where did the 1918 virus originate? (ii) Was there something unique about the genetic structure of the virus which enabled it to achieve such a high killing potential? (iii) Were the geographical circumstances of 1918 unique with so many young men

travelling the world in troopships? (iv) Was the 1918 virus pantropic like H5 influenza in chickens where it infects all organs including the alimentary tract and nervous system or did it confine its virulence to the respiratory tract?

There is a lack of consensus in the literature as to the genetic origin of the 1918 pandemic influenza virus. Langford (2005) posits from historical records that that 1918 pandemic influenza virus originated in China. The basis of Langford's hypothesis is the lower mortality rates of Chinese during the 1918 influenza pandemic. Lower mortality rates may have been the result of Chinese having prior exposure to earlier mutations of the virus and hence some immunity to the virulent strain (Langford, 2005). Langford further posits the travel routes of Chinese workers to France and other countries during the period may explain the global spread of the 1918 influenza pandemic virus. Reid et al. (2004) used phylogenetic analysis data of gene sequences from the 1918 influenza pandemic virus for investigating the origin of the virus(es).

Formalin-fixed-paraffin-embedded lung tissue from victims of the 1918 pandemic and exhumed victims of the outbreak in the Artic regions frozen case material has made it possible to characterize the genetic sequences of the 1918 influenza pandemic virus (Oxford, 2000; Reid, Taubenberger, & Fanning, 2004). Sequencing of the RNA fragments identified the 1918 influenza pandemic virus as a novel H1N1 influenza A virus (Keeler, 2011). Reid et al. (2004) suggested emerging data about the genetic origins of the 1918 H1N1 pandemic strain indicate some of the genome segments might have a novel origin that has not been isolated in strains responsible for other pandemics. Morens and Fauci (2007) attested genetic sequencing identifies the ultimate ancestral source of

the 1918 H1N1 pandemic virus to be avian. However, this does not explain if there something unique about the genetic structure of the virus which enabled it to achieve such a high killing potential (Oxford, 2000).

Current global death toll estimates from the pandemic Spanish flu of 1918 is at least 40 million persons and 500 million infected (Oxford, 2000; Reid et al., 2004; Tumpey, et al., 2005). An estimated death in the United States from pandemic Spanish flu of 1918 is 675,000 persons (Tumpey, et al., 2005). Frost (1919) investigated and compared the mortality rates resulting from influenza and pneumonia disease from the late nineteenth and early twentieth centuries. The investigation by Frost focused on the mortality rates in major U.S. cities during that time period. Morens and Fauci (2007) further investigated the mortality rates caused by the pandemic Spanish flu of 1918.

The mortality rates of the pandemic Spanish flu of 1918, unlike seasonal outbreaks, shows a "W" shape mortality curve (Morens & Fauci, 2007). During most influenza epidemics and pandemics, the mortality curve is "U" shape where the highest mortality rates are seen in the very young and very old with lower mortality rates seen in other age groups (Keeler, 2011). Morens and Fauci (2007) reported that excess mortality in persons 20 – 40 years of age resulting from the pandemic Spanish flu of 1918 produced the "W" shape mortality curve. Further, Morens and Fauci asserted the extradordinary excess influenza mortality in persons 20 – 40 years of age from the pandemic Spanish flu of 1918 may be the most important unsolved mystery of the pandemic.

Starko (2009) investigated possible causes of the excess influenza mortality in persons 20 – 40 years of age from the pandemic Spanish flu of 1918. In this study, it was hypothesized that salicylate (aspirin) therapy for influenza during the pandemic Spanish flu of 1918 resulted in toxicity and pulmonary edema, which contributed to the incidence and severity of severe acute respiratory distress (ARDS) – like condition, subsequent bacterial infection, and overall mortality (Starko, 2009). Starko reasoned the combination of the loss of Bayer's patent on aspirin in February of 1917, and the aspirin regimens (dose and schedule) recommended in 1918 contributed to the excess mortality rate. The loss of Bayer's patent on aspirin in February of 1917 opened the aspirin market to many manufacturers, and the aspirin regimens in 1918 are now known to regularly produce toxicity (Starko, 2009). Thus, toxic aspirin regimens in combination with pandemic Spanish flu of 1918 may have resulted in increased mortality among 20 – 40 year olds. Unlike Starko who hypothesized salicylate therapy to exacerbate the mortality, Tumpey, et al. (2005) investigated the high mortality rate using a reconstructed 1918 Spanish influenza pandemic virus.

Tumpey, et al. (2005) generated a virus containing the complete coding sequences of the eight viral gene segments from the 1918 Spanish influenza pandemic virus. The purpose of this effort was to understand the molecular basis of the virulence of the 1918 Spanish influenza pandemic virus (Tumpey, et al., 2005). To evaluate the pathogenecity of the 1918 Spanish influenza pandemic virus, the researchers of the Tumpey, et al. study intranasally inoculated BALB/c mice with two independently generated 1918 viruses and then determined morbidity, virus replication, and 50% lethal dose titers.

The histopathology of lung tissue from intranasally inoculated BALB/c mice showed similarity to lung tissues from human cases who died from primary influenza pneumonia in 1918 (Tumpey, et al., 2005). Further, the researchers of the Tumpey, et al. (2005) study were able to show the 1918 Spanish influenza pandemic virus HA and polymerase genes were associated with increased virulence in chicken embryos and also the 1918 Spanish influenza pandemic virus replication rate is significantly greater than that of the contemporary human Tx/91 (H1N1) virus. However, studies of the virulence of the 1918 Spanish influenza pandemic virus have been unable to explain the three waves in the 1918-1919 pandemic.

Morens and Fauci (2007) reported the pandemic Spanish flu of 1918 spread in three rapidly recurring waves within a nine month period. Keeler (2011) reported the three waves occurred over sixteen months. This rapid spread was unprecedented at the time (Keeler, 2011). The first wave or Spring wave began in March 1918 and spread heterogeneously through the United States, Europe, and possibly Asia over a six month period (Langford, 2005; Taubenberger & Morens, 2009). The second wave or Fall wave appeared in August 1918 in a much more severe form than in the first wave (Keeler, 2011; Langford, 2005). This second wave of August 1918 apparently began in three locations: Freetown, Sierra Leone; in Brest, France; and in Boston, Massachusets (Langford, 2005). The third wave appeared in many nations during the early part of 1919 (Langford, 2005; Taubenberger & Morens, 2009).

By the end of the pandemic, approximately one-third of the world population was infected and death estimates between 50 – 100 million persons (Keeler, 2011). Mortality

from the pandemic Spanish flu of 1918 was highest during the second wave, followed by the third wave, and then the first wave (Taubenberger & Morens, 2006). The identity of 1918 pandemic virus was completed using formalin fixed lung tissue from more than 80 victims of the second wave (Oxford, 2000). The identity of the viral varients of the first and third waves is still unknown (Morens & Fauci, 2007). Another unexplained phenomena is the unprecedented velocity of global spread of the pandemic Spanish flu of 1918.

It is widely accepted in the literature the pandemic Spanish flu of 1918 spread throughout the globe in less than a two year period. Oxford (2000) studied the geographical circumstances of troopship movements and wartime efforts are asserted to play an important role in supporting the global spread of the 1918 Spanish influenza pandemic virus. Patterson and Pyle (1983) investigated the diffusion of the pandemic Spanish flu of 1918 across sub-Saharan Africa. In the Patterson and Pyle study, the data analyzed primarily came from published government reports and a few contemporary articles. The authors acknowledged the reporting of data to be spotty; where times and places of some outbreaks were missing (Patterson & Pyle, 1983). Patterson and Pyle asserted the devastation of the second wave of the 1918 – 1919 pandemic was totally unexpected, unexplainable, and uncontrollable in Africa and thus, more useful data would not greatly modify the conclusions of the study.

The second wave of the pandemic Spanish flu of 1918 has been reported to have appeared in Sierra Leone in August 1918 (Langford, 2005). Patterson and Pyle (1983) reported that Freetown, Sierra Leone was the first place in sub-Sarahan Africa where the

"Fall wave" hit and subsequently spread to all West Africa. Patterson and Pyle further asserted that wartime disruption with the movement of troops and laborers, and the newly created colonial transportion network are two factors that helped spread the disease.

There are several common phenomena to the spread and spatial diffusion of infectious diseases. These common phenomena underpin the research conclusions of the Patterson and Pyle (1983) study. These spatial diffusion phenomena are: (i) extremely virulent agents can penetrate areas rapidly (especially when there are low immunity levels) and linear pathways result; (ii) radical spatial diffusion takes place from the onset, with the disease spreading to places adjacent to the major pathways and beyond; and (iii) these forms of diffusion often coalesce into 'clinical fronts', and, in many instances, spatial-temporal diffusion waves can be identified (Patterson & Pyle, 1983). Patterson and Pyle concluded the research findings support pandemic disease diffusion along transportation routes. Additionally, interval-scaled time-distance plots indicate the velocity of movement along the South Africa/Congo pathway to be much more rapid than the pathways of the Gold Coast, Nigeria, and Benin (Patterson & Pyle, 1983). By applying Spearman Rank-Order Correlation (R_S) to the interval-scaled time-distance plot data, the researchers of the Patterson and Pyle study were able to estimate pandemic disease spread velocities. Based on these calculations, Patterson and Pyle asserted the pandemic did not originate in Africa, and that West and East Africa were infected separately.

The second wave of the pandemic disease ended in the 1918 summer - fall season and the third wave of the pandemic disease ended after the 1918-1919 winter season

(Morens & Fauci, 2007). Taubenberger and Morens (2006) posit the virus may have shifted due to confrontation by selection pressures of population immunity. Thus, the pandemic viruses begin to drift genetically and eventually settle into a pattern of annual epidemic recurrences (Taubenberger & Morens, 2006). Nearly 40 years following the emergence of the 1918 Spanish influenza pandemic virus, an H2 subtype of influenza A virus emerged in the Yunan Province of China in February 1957 (Oxford, 2000).

1957-1958 (Far East Flu)

The influenza pandemic of 1957-1958 first occurred in the Yunan Province of China in February of 1957 (Potter, 2001). The manuscript by Payne (1958) included an early account of the speed and spread of the 1957-1958 pandemic. From the original outbreak in the Yunan Province, the virus spread throughout the world within seven months (Payne, 1958). Payne reported that in 1957, initial virus isolation was performed in four laboratories: Chanchung, China by Chu et al.; Peking, China by Tang and Liang; Singapore by Hale; and at a United States Army laboratory in Japan. The 1957 pandemic influenza virus was an H2N2 strain (Pappas, et al., 2010). Webster et al. (1992) reported the1957 pandemic influenza virus H2N2 strain originated as a reassortant of PB1, HA, and NA gene segments. Lindstrom et al. (2004) further reported the PB1, HA, and NA gene segments of the 1957 pandemic influenza virus H2N2 were avian origin and the remaining five gene segments were of human H1N1 origin.

The 1957 pandemic influenza virus H2N2 developed considerable genetic variability as the epidemic spread throughout the globe (Lindstrom, Cox, & Klimov, 2004). A few of the 1957 pandemic influenza virus H2N2 sub lineage strains include:

A/Albany/22/1957, A/Japan/305-/1957, A/Guiyang/1/1957, and A/RI/5+/1957. Xu, McBride, Paulson, Basler, and Wilson (2010) investigated avian, human, and swine viral isolates from 1957 H2N2 pandemic to better understand the 1957 pandemic influenza viral genetic sequencing and molecular structures.

Phylogenetic analysis of 52 viruses isolated between 1957 and 1968 was conducted by Lindstrom et al. (2004). From this investigation, Lindstrom et al. asserted the 1957 pandemic influenza viruses diverged into two distinct clades from one common ancestor. It is pausible the rapid global spread and increased mortality from the 1957 pandemic influenza virus was a result of the genetic diversity of the H2N2 viral sublineages. Therefore, human populations without previous exposure to viral strains the humoral immunity and response (antibody-mediated system) would be challenged.

The 1957 pandemic influenza viruses circulated for only 11 years from 1957 to 1968 (Krause, et al., 2012). The mortality from the 1957 H2N2 pandemic is estimated to be 2 million deaths globally (Pappas, et al., 2010). Potter (2001) reported deaths due to the 1957 H2N2 pandemic were calculated at approximately 80,000 in the United States. Simonsen, Clarke, Schonberger, Arden, Cox, and Fukuda (1998) examined age-specific mortality related to the 1957 H2N2 pandemic in the United States. The researchers of the Simonsen et al. (1998) study calculated the proportion (%) of excess deaths in persons < 65 years old as: [1957-1958 = 36%], [1959-1960 = 28%], [1962-1963 = 26%], and [1967-1968 = 4%]. During the 1957-1958 H2N2 pandemic (Far East Flu), persons < 65 years old accounted for 36% of all excess influenza-realted deaths which was lower than during the 1968-1969 H3N2 pandemic (Hong Kong Flu) where persons < 65 years old

accounted for 48% of all excess influenza-related deaths (Simonsen, Clarke, Schonberger, Arden, Cox, & Fukuda, 1998).

1968-1969 (Hong Kong Flu)

In mid-July, 1968, a wide spread outbreak of acute respiratory disease occurred in Southeast China. This outbreak was reported in *The Times of London* (Cockburn et al., 1969). Human-to-human transmission rates of this acute respiratory disease were high. By the end of July 1968, an estimated one-half a million persons had become infected (Keeler, 2011). This rapidly spreading influenza disease became known as the "Hong Kong flu". Cockburn, Delon, and Ferreria (1969) reported the "Hong Kong flu" first appeared in the United States in California during October 1968. By the end of December 1968, "Hong Kong flu" infections were reported across the U.S. in nearly every state (Cockburn et al., 1969). The disease also spread to Australia, India, and Iran by mid-August 1968 (Keeler, 2011). Further, Keeler (2011) reported the disease spread to Europe by September 1968 and the global epidemic was reported in nearly every country by January 1969.

Even though the majority of people infected by the "Hong Kong flu" experienced typical flu-like symptoms, high mortality rates were reported (Keeler, 2011). Simonsen et al. (1998) reported that during the 1968-1969 "Hong Kong flu" pandemic, persons < 65 years old accounted for 48% of all influenza-realted excess deaths. The findings of this study is in contrast to the recent work by Keeler (2011). Keeler reported high mortality rates were observed in the very young and the very old due mainly to pneumonia caused by secondary infections. This "smoldering" influenza disease was the subject of

inconsistencies in the news media as well. In the January 18, 1969 publication of the British Medical Journal, a correspondence by F. Desmond MacCarthy was included. MacCarthy (1969) wrote:

> Sir, I am surprised no one has written to you before concerning the hysterical outburst by the press and the B.B.C. with regard to this subject. Every day there is a head line. We must expect a severe epidemic or not; we should be vaccinated or cannot be because there is no vaccine available; thousands of cases in America and hundreds of deaths; or, it's not very serious, no worse than previous epidemics; 250,000 doses of vaccine released next week. Cannot the press and the B.B.C. be controlled or at least persuaded to behave more responsibly in a matter which affects every family doctor, and, by the unnecessary anxiety they have created, increases their work tremendously? (p. 182).

The "Hong Kong flu" has been described as a "smoldering" pandemic because the first wave (1968 – 1969) resulted in high mortality rates in the United States, and the second wave (1969 – 1970) resulted high mortality rates in Europe and Asia (Keeler, 2011; Viboud, Grais, Lafont, Miller, & Simonsen, 2005). Viboud, Grais, Lafont, Miller, and Simonsen (2005) described this phenomena as "counterintuitive" since a novel virus introduced into a susceptible population should demonstrate decreasing impact over time as population immunity increases.

The novel virus responsible for the "Hong Kong flu" pandemic is A/HongKong/68 (H3N2) (Viboud et al., 2005). The literature supports the hypothesis that A/HongKong/68 (H3N2) resulted from a reassortment event between a circulating human

H2N2 virus and an avian influenza virus, acquiring novel HA (H3 subtype) and PB1 gene segments (Lindstrom et al., 2004; Taubenberger & Kash, 2010; Taubenberger & Morens, 2006; Viboud et al., 2005). Taubenberger and Kash (2010) proclaimed the six other gene segment of the A/HongKong/68 (H3N2) were retained from the 1957 – 1958 H2N2 pandemic virus.

Unlike the 1957 – 1958 H2N2 virus which circulated for 11 years, the 1968 – 1969 H3N2 virus continues to be isolated at low levels around the globe (Taubenberger & Kash, 2010). Lindstrom et al. (2004) suggested diversity at the protein level among early H3N2 viruses is an explanation why surviving H3N2 viruses to possess a number of amino acid changes. These amino acid changes thus render these H3N2 virus strains more fit (Lindstrom et al., 2004). Less fit is the A/New Jersey/76 (HswlN1) virus. A/New Jersey/76 (HswlN1) virus is responsible for the 1976 influenza outbreak at Fort Dix, NJ and was only identifiable in circulation for approximately three months (Gaydos et al., 2006).

1976 Pandemic Flu Threat (Fort Dix Swine Flu)

During the months of January and February 1976, novel swine influenza A virus outbreak occurred in Fort Dix, New Jersey. More than 230 soldiers were affected of which 13 soldiers (12 basic trainees and one cadre office worker) developed severe respiratory illness with one death (Gaydos et al., 2006). Kendal, Goldfield, Noble, and Dowdle (1977) described the laboratory procedures of identification and preliminary antigenic analysis of the novel swine influenza A virus outbreak at Fort Dix, New Jersey.

A collaborative effort by the New Jersey Department of Health and the World Health Organization Collaborating Center at the Center for Disease Control identified the novel virus and designated it A/New Jersey/76 (HswlN1) influenza virus (Kendal, Goldfield, Noble, & Dowdle, 1977). Kendal et al. (1977) asserted the A/New Jersey/76 (HswlN1) influenza virus was transmitted from swine to man. Top and Russell (1976) further suggested a new recruit introduced A/New Jersey/76 (HswlN1) to Fort, Dix, NJ early in 1976 after the holidays. Gaydos et al. (2006) speculated human-to-human transmission of A/New Jersey/76 (HswlN1) occurred because of the close contacts in the unique basic training environment, with limited transmission outside the basic training environment. Further, Gaydos et al. (2006) suggested the Fort Dix outbreak may have been a zoonotic anomaly caused by introduction of an animal virus into a stressed population in close contact in crowded facilities during a cold winter.

A seroepidemiologic study of basic combat training trainees at the time of the Fort Dix, NJ outbreak strongly supported human-to-human transmission among the contacts of the case (Hodder, Gaydos, Allen, Top, Nowosiwsky, & Russell, 1977). Interestingly, the A/New Jersey/76 (HswlN1) influenza outbreak was limited to Fort Dix, NJ, and was no longer isolated after March 19, 1976 (Gaydos et al., 2006). It has been hypothesized in the literature that a co-circulating and widely prevalent in the civilian population seasonal influenza virus, Influenza A/Victoria may have been in competition with A/New Jersey/76 (HswlN1) (Top & Russell, 1976). Top and Russell (1976) speculated the continual introduction of A/Victoria virus into the reception center at Fort Dix, NJ, in contrast to the limited introduction of A/New Jersey/76 (HswlN1) virus may

have resulted in a gradual reduction in the possibility of individual exposure to A/New Jersey/76 (HswlN1) virus and a greater possibility of exposure to A/Victoria virus. A little over a year later, in 1977, influenza A subtype of H1N1 emerged in China (Kung et al., 1978).

1977 Pandemic Flu Threat (New Flu Virus in Northern China, aka Russian Flu, 1977)

By the end of May and the beginning of June 1977, another outbreak of influenza was identified in Liaoning Province and Tientsin Municipality in China (Kung et al., 1978). Also known as the 1977 Russian flu, this outbreak is often considered the fourth pandemic of the twentieth century (Keeler, 2011). Kung, Jen, Yuan, Tien, and Chu (1978) reported sera samples were collected from four different areas of China and submtted to Peking for identification. The influenza was identified to be H1N1 (Oxford, 2000). During this outbreak, the disease concentration was around middle and primary schools (Kung et al., 1978). Oxford (2000) reasons persons over twenty years possessed antibodies to the virus so illness was limited mostly to children and adolescents.

The Peking Health and Anti-epidemic Station conducted a survey immediately following the July – August epidemic. The Peking Health and Anti-epidemic Station surveillance revealed mortality was highest in people aged 7 – 12 years and 13 – 20 years, and was notably lower in those aged 21 – 30 years and further declined thereafter (Kung et al., 1978). Further, Kung et al. (1978) reported the 1977 H1N1 pandemic virus spread rather slowly. The virus first emerged in Liaoning in May and covered the whole province by August, and spread to different parts of China gradually between June and

October (Kung et al., 1978). In contrast to Kung et al., Oxford (2000) described the spread of the 1977 H1N1 pandemic virus as rapid; causing outbreaks in Russia and 7 – 9 months later causing outbreaks in the United Kingdom, United States, Europe, and Australia.

Phylogenetic analysis of the 1977 H1N1 pandemic virus showed genetic and antigenetic similarity to previously identified strains circulating in the 1950s (Keeler, 2011; Oxford, 2000). The genetic and antigenetic similarities to previously circulating influenza A viruses may provide insight and explanation as to why mortality was highest in people aged 7 – 12 years and 13 – 20 years. Persons older than 20 years may have had pre-existing humoral immunity to the 1977 H1N1 pandemic virus. Kung et al. (1978) asserted that an ancestral H1N1 virus was preserved either in the form of a latent infection in certain human individuals, or in some animal host. Further, Kung et al. posit the reappearance and epidemicity could be explained either by reactivation of the latent virus, reacquisition of human virulence, or from the opportunity for transmission to a susceptible human being. Oxford (2000) contended the 1977 H1N1 pandemic virus re-emerged from a laboratory freezer by error. Keeler (2011) upheld the Oxford contention by suggesting the virus release was the result of accidental laboratory release or some type of experimentation with influenza vaccination.

1996 Pandemic Flu Threat (Avian Flu H5N1)

In 1996, the highly pathogenic avian influenza virus H5N1 (HPAI H5N1) emerged in southern China. This influenza A virus caused a moderate number of deaths in geese in the Guangdong region of China (Webster et al., 2006). Webster, Peiris, Chen,

and Guan (2006) reported that this goose virus acquired internal gene segments found in quail (A/Quail/HK/G1/97 [H9N2]) and also the neuraminidase gene segment from the duck virus (A/Teal/HK/W312/97 [H6N1]) before becoming widespread in the live bird markets of Hong Kong. This highly pathogenic avian influenza A (H5N1) virus decimated three chicken farms in the new Territories Hong Kong in March 1997 (Hatta & Kawaoka, 2002). Following this event, an epizootic outbreak of HPAI H5N1 occurred in Hong Kong that resulted in 18 human cases and six deaths (Taubenberger & Morens, 2009; Webster et al., 2006).

The genetic nature of HPAI H5N1 RNA results in a relatively high rate of nucleotide substitutions (Pfeiffer, Otte, Roland-Holst, Inui, Tung, & Zilberman, 2011). These nucleotide substitutions result in point mutations, a process known as "antigenic drift" (Aldras, 2011). The literature suggests that antigenic drift is the principle reason why particular influenza strains continue to circulate (Pfeiffer et al., 2011). Six years after the epizootic outbreak in China, HPAI H5N1 reappeared in epizootic form and spread widely throughout Southeast Asia (Taubenberger & Morens, 2009).

The first reported human case of avian influenza A (H5N1) infection was a three year old boy (Hatta & Kawaoka, 2002). Hatta and Kawaoka (2002) reported that a three year boy began to show influenza-like-illness in May 1997, and died on day 16 of extensive influenza-related pneumonia complicated by Reye's syndrome. By the end of 1997, a total of 18 Hong Kong residents had become infected with avian influenza A (H5N1) virus including the six fatalities (Hatta & Kawaoka, 2002). Hatta and Kawaoka reported the RNA gene segments isolated from the human cases were identical to the

RNA gene segments isolated from the live bird markets of Hong Kong. Thus, this avian influenza A (H5N1) virus was able to cross the species barrier without genetic reassortment with human viruses (Hatta & Kawaoka, 2002). The mass culling of all domestic poultry in Hong Kong eradicated this avian influenza A (H5N1) virus and the genotype has not been detected since that time (Webster et al., 2006). An estimated 1.5 million poultry were culled in Hong Kong (Yuen & Wong, 2005). However, various reassortments of the virus continue to emerge from goose and duck reservoirs resulting in animal-to-human transmission.

In February 2002, a descendant of the 1997 avian influenza A (H5N1) virus infected two persons of whom one died (Webster et al., 2006). Webster et al. (2006) reported that by the end of 2002, this single H5N1 genotype was responsible for killing most wild, domestic, and exotic waterfowl in Hong Kong nature parks. This virus was the precursor of the Z genotype which rapidly spread across Southeast Asia, affecting Vietnam, Thailand, Indonesia, Cambodia, Laos, Korea, Japan, China, and later Malaysia (Webster et al., 2006).

In late April 2005, an avian influenza A (H5N1) outbreak occurred at Qinghai Lake, China (Chen, et al., 2005). Chen, et al. (2005) reported the mortality rate of birds was more than 100 per day during the outbreak. An estimated 1,500 bird deaths resulted of this outbreak (Chen, et al., 2005). Further, Chen, et al. reported 90% of the affected birds were bar-headed geese, and the remainder being brown-headed gulls and great black-headed gulls. Kou, et al. (2009) reported worldwide decrease of bar-headed goose population decreased by 5% - 10% resulting from this epizootic disease alone. Virus

sequencing comparisons of samples taken from the Qinghai outbreak revealed the avian influenza A (H5N1) viruses were almost identical across the whole genome (Chen, et al., 2005). The authors of the Chen, et al. study asserted the phylogenic analysis of the Qinghai viral samples showed a close relationship to the avian influenza A (H5N1) viruses isolated from the poultry markets in Fujian, Guangdong, Hunan, and Yunnan provinces in 2005.

In the Wan, et al. (2005) study, whole genome sequencing and phylogenic analysis of four strains of avian influenza A (H5N1) virus isolated from different territory areas [A/Duck/Guangdong/173/04 (H5N1) (Dk/GD/173/04 in brief, Central Guangdong), A/Chicken/Guangdong/174/04 (H5N1) (Ck/GD/174/04 in brief, Western Guangdong), A/Chicken/Guangdong/178/04 (H5N1) (Ck/GD/178/04 in brief, Eastern Guangdong), and A/Chicken/Guangdong/191/04 (H5N1)(Ck/GD/191/04 in brief, Northern Guangdong)] was completed. Wan, et al. asserted the results of this investigation demonstrated the four viruses retained most of the reported H5N1 AIV sequence properties relevant to virus virulence and host adaptation. Kou, et al. (2009) mentioned that prior to 1997, it was believed that avian influenza virus could not host adapt to humans until the genes of avian influenza virus mixed with those of human viruses, which was deduced by swine-Mixing Vessel Theory-the intermediate host. The avian influenza A (H5N1) outbreak which has been traced back to March of 1996 in the Guangdong region of China dispelled this belief.

China is in a critical position along the migration flyways for migratory birds across Eurasia which may provide explanation for the unprecendented spread and human

cases virus infection (Kou, et al., 2009). World Health Organization (2004) reported in December 2003 and outbreak of avian influenza A (H5N1) occurred in the Republic of Korea and on January 12, 2004 Japanese authorities announced the death of approximately 6000 chickens at a single poultry farm due to infection from a single strain of the same virus. For the period 2004-2005, 88 human cases with 55 deaths (overall case-fatality rate of 58%) of H5N1 virus infection have been reported in Cambodia, Thailand, and Vietnam (Yuen & Wong, 2005). For the same period, Yuen and Wong (2005) reported an estimated 3.835 million poultry have been affected or destroyed in the Hong Kong Special Administrative Region. By November 2005, the World Health Organization reported 133 human cases of H5N1 AIV in Southeast Asian countries with a case fatality rate approximately 50% (Spicuzza, Spicuzza, La Rosa, Polosa, & Di Maria, 2007).

As of April 17, 2008 the World Health Organization reported 381 human cases of avian influenza A (H5N1) virus infection with 240 deaths from 15 countries (Australian Nursing Journal, 2008). At the time of this writing there are no confirmed case of human-to-human transmission of avian influenza A (H5N1) virus. However, a review of the literature revealed possible events of human-to-human transmission of avian influenza A (H5N1).

Ungchusak, et al. (2005) investigated a possible human-to-human transmission of avian influenza A (H5N1) in a family cluster. The index patient was an 11 year ago girl who lived with her aunt and became ill three to four days following exposure to dying household chickens (Ungchusak, et al., 2005). Ungchusak, et al. indicated the index

patient's mother came from a distant city, had no recognized exposure to poultry, provided care for her in the hospital, and died from pneumonia after providing 16 to 18 hours of unprotected nursing care. Additionally, the aunt also provided unprotected nursing care; had fever five days after the mother first had fever, followed by pneumonia seven days later (Ungchusak, et al., 2005). Ungchusak, et al. reported autopsy tissue from the mother and nasopharyngeal and throat swabs from the aunt were positive for avian influenza A (H5N1) by RT-PCR. The Australian Nursing Journal (2008) reported the Chinese Center for Disease Control and Prevention has investigated the case of a 24 year old man and his 52 year old father diagnosed with avian influenza A (H5N1) within a week of each other in the Jiangsu Province in China December 2007. The son's exposure to avian influenza A (H5N1) was attributed to a poultry market six days prior to the onset of illness, and the father's exposure is attributed to unprotected exposure to his ill son (Australian Nursing Journal, 2008).

The World Health Organization requires member states to report every sporadic case of H5N1 human infection (World Health Organization, 2013). During December of 2012, Egypt confirmed its 169th human case of H5N1 in a 2-year-old girl, and Indonesia confirmed its 192th human case of H5N1 in a 4-year-old boy (World Health Organization, 2012). A challenge to monitoring H5N1 human cases is confirming true-positive infections. Rapid diagnostic tests for influenza A virus identification have been developed. However, even though most rapid tests are more than 70% sensitive and more than 90% specific, this yields a 30% false-negative sample result (Shive, 2011b).

A cohort study for serologic detection for anti-H5 antibody was conducted shortly after the HPAI H5N1 Hong Kong outbreak of 1997 (Katz, et al., 1999). In this study, household and non-household contacts of the 18 human case of H5N1 were investigated for antibody response and exposure to poultry. The results of this study showed only seven of the 124 persons studied were serologically positive for H5 antibody. The researchers concluded that of the seven cases, only one H5 antibody-positive household contact with no exposure to poultry was evidence for human-to-human transmission (Katz, et al., 1999).

To date, avian influenza A (H5N1) has not shown to be effectively transmitted from person to person, however, the high case fatality rate in Southeast Asia is alarming (Yuen & Wong, 2005). Ungchusak, et al. (2005) asserted the reassortment events of avian influenza A (H5N1) since its emergence in China in 1997, suggests the virus may become more efficient in infecting humans, either by acquiring genetic material from a human influenza virus through reassortment or by adapting its receptor binding sites. Chen, et al. (2005) forewarned that if current strains of avian influenza A (H5N1) become established in bar-headed geese, there is the danger the virus may be carried along the birds' wintering migratation routes to densely populated areas in the South Asian subcontinent. This event could possibly result in further distribution of avian influenza A (H5N1) along the flyways to Europe which would vastly expand the global distribution of H5N1 (Chen, et al., 2005). At the time of this writing the WHO reported there have been a total of 637 cases of avian influenza A virus infection with 378 deaths. There are

no reported cases of avian influenza A virus infection in North, Central, or South America (World Health Organization, 2013a).

Animal-to-human transmission is considered "primary" transmission and human-to-human transmission of zoonotic diseases is considered "secondary" transmission (Lloyd-Smith, et al., 2009). The literature recognizes personal hygiene and vaccination as the most important and effective methods for controlling for spread of influenza viruses (McClure, Gleason, & Brenner, 2011). Mao and Yang (2012) posit human networks, infectious diseases, and human preventive behavior are intrinsically inter-related. Personal hygiene is an extension of human preventive behavior for controlling the spread of influenza viruses. Avoidance of animal-human contact with dead or sick birds is the best prevention for preventing H5N1 infection (Centers for Disease Control and Prevention, 2010). Vaccination is another approach to zoonotic disease control.

Vaccine development is a process which can take months to years to develop and produce ample supply for national distribution (Hessel, 2011). Vaccine production and availability cannot guarantee full compliance by all individuals. One group of individuals is pregnant women. A recent study found that 80% of pregnant women believed flu vaccines could cause birth defects and only 50% of the women were aware of the national recommendations for vaccination during pregnancy (Durica, Murchison, & Weiss, 2011). Another approach to zoonotic disease control is surveillance.

A predominance of the monitoring and control of highly pathogenic avian influenza H5N1 (HPAI H5N1) has been accomplished by cloacal swab sampling of migratory birds and monitoring for morbidity and mortality events. This process is labor

intensive, and morbidity and mortality trending tends to strongly bias towards birds which are susceptible to the virus (Farnsworth, et al., 2011). Migratory birds that are asymptomatic can spread the virus across long distances including national borders (Gaidet, et al., 2010). Control measures for outbreaks of avian influenza have included the depopulation of commercial birds and flocks. During the October 1984 HPAI H5N2 outbreak in Pennsylvania, more than 17 million birds were depopulated (Okonkwo & Lupankwa, 2011). Oyana et al. (2011) estimated between 2004 to 2008, nearly 29 million birds had been culled in China in an effort to control HPAI H5N1.

The review of the literature about the topic of the current avian influenza A (H5N1) pandemic revealed the seriousness and global concern as a major pandemic threat to humans. As of date, the current WHO phase of pandemic alert for avian influenza A (H5N1) is: ALERT (World Health Organization, 2013). The potential for further spread of avian influenza A (H5N1) virus and the high case fatality rate warrants further surveillance.

A different approach to monitoring and controlling for HPAI H5N1 human cases would be water monitoring for HPAI H5N1 viruses. As previously noted, the review of the literature identified research studies that support migratory waterfowl as the natural reservoir for influenza A viruses (Webster et al., 1992). Further, the review of the literature identified research studies that support fecal-oral transmission of influenza A viruses between waterfowl (Franklin et al., 2011). Therefore, geographical water analysis-where waterfowl are found-for influenza viruses may be an approach to monitoring and controlling for HPAI H5N1 strains.

An environmental monitoring approach could provide public health officials with geographic specific data for influenza intervention and response efforts. Additionally, by not culling flocks of birds, evolutionary pathways may develop poultry lineages that are HPAI H5N1 resistant. For these reasons, in this dissertation study, I investigated the burden of avian influenza A (H5N1) in rural and urban artifical recirculating water ponds. While global public health efforts have focused on controling HPAI H5N1, a swine flu H1N1 outbreak occurred in 2009.

2009-2010 (Swine Flu H1N1 in United States)

In Mid-March of 2009, Mexican health surveillance began identifying cases of influenza-like-illness during a time when seasonal outbreaks are usually declining (World Health Organization, 2009a). World Health Organization (2009a) reported Mexican health officials suspected these phenomena to be an outbreak of a newly emerged influenza virus. Shortly after, the newly emerged influenza virus was detected in the United States. By mid-April 2009, two children in Southern California developed febrile respiratory illness caused by infection from genetically similar swine virus (Centers for Disease Control and Prevention, 2009). The Centers for Disease Control and Prevention (2009) reported this virus contained a unique combination of gene segments that had not been previously reported among human or swine influenza viruses in the United States or internationally.

Mexican health officials continued enhanced surveillance and by April 27, 2009 reported 26 confirmed cases with 7 deaths, 286 probable cases and 1551 suspect cases with 84 deaths (World Health Organization, 2009a). By mid-May, 11,932 suspected

cases, 949 laboratory confirmed cases of novel influenza A (H1N1) infection, including 41 deaths were reported in Mexico (Centers for Disease Control and Prevention, 2009). Two possible human cases were reported in Guadalupe, Texas by mid-April 2009 (Centers for Disease Control and Prevention, 2009). The novel influenza A (H1N1) virus quickly spread across the United States.

In early May 2009, the Centers for Disease Control and Prevention (2009) reported 1,487 confirmed (n = 642) and probable cases (n = 845) had been reported in 43 states. The novel influenza A (H1N1) virus began appearing globally. By early May 2009, 309 laboratory-confirmed cases had been identified in 21 countries other than the United States and Mexico (Centers for Disease Control and Prevention, 2009). This novel influenza A (H1N1) virus demonstrated efficient and rapid spread across the human population.

By the end of May 2009, nearly 13,000 novel influenza A (H1N1) virus infection cases had been reported in over 40 countries (World Health Organization, 2009b). By mid-June 2010, the CDC reported approximately 740,000 samples were tested for influenza, and the number of laboratory-confirmed positives was approximately four-times the average for the previous four seasons (Centers for Disese Control and Prevention, 2010). The rapid spread across the human population was enhanced by antigenic drift of the novel influenza A (H1N1) virus.

The novel influenza A (H1N1) virus is a reassortant of swine influenza A viruses from North American and Eurasian lineages (H1N1, H1N2 and/or H3N2) which has gene segments originating from swine, human and avian influenza A viruses (World Health

Organization, 2009b). The 2009 H1N1 reference vaccine virus selected by the WHO was the A/California/7/2009 (Centers for Disease Control and Prevention, 2010). The Centers for Disease Control and Prevention (2010) reported that from antigenic characterization of 944 viruses that were 2009 H1N1, 942 (99.8%) were related to the A/California/7/2009 virus. Interestingly, a mutation of the novel influenza A (H1N1) 2009 virus was suspect to be resistant to the oral antiviral drug, Tamiflu (Genetech - oseltamivir phosphate).

In mid-July 2009, two adolescent girls, both receiving oseltamivir chemoprophylaxis after exposure to a person with influenza like illness (ILI), became ill (Centers for Disease Control and Prevention, 2009). The Centers for Disease Control and Prevention (2009) reported that influenza samples collected from both adolescent girls showed the presence of pandemic 2009 influenza A (H1N1) virus and similar genetic mutations. These findings are suggestive of evolutionary development of an antiviral resistent 2009 influenza A (H1N1) virus. Although pandemic 2009 influenza A (H1N1) virus infection causes mild disease disease, severe illnesses resulting in hospitalization and death have occurred in many countries, including: Canada, Costa Rica, Mexico, and the United States (World Health Organization, 2009b).

Globally, pandemic 2009 influenza A (H1N1) virus infection has been found to cause a disproportionately higher rate of hospitalization and death among indigenous populations from Australia, Canada, and New Zealand (Centers for Disease Control and Prevention, 2009). Indigenous populations in the United States have also shown a disproportionately higher rate of hospitalization and death from pandemic 2009 influenza

A (H1N1) virus infection. The Centers for Disease Control and Prevention (2009) reported American Indian/Alaskan Natives had H1N1 mortality rates four times higher than persons in all other racial/ethnic populations combined.

The increased mortality among the indigenous people of the United States may be due to social injustice. These social injustice conditions may include: high prevalence of chronic health conditions (e.g., diabetes and asthma) among American Indian/Alaskan Natives that predisposes them to influenza complications, poverty (e.g., poor living conditions), and delayed access to care (Centers for Disease Control and Prevention, 2009). The Centers for Disease Control and Prevention (2009) recommended increased efforts to increase awareness among American Indian/Alaskan Natives and their health-care providers of the potential severity of influenza and current recommendations regarding the timely use of antiviral medications. This dissertation study may bring about positive social change among the American Indian/Alaskan Native people of the United States.

In this dissertation study, I investigated the burden of influenza A virus in aquatic environments in urban and rural community settings. The aquatic environment is understood in the literature to be a human-animal interface for influenza A virus (Franklin et al., 2011; Lebarbenchon & Stallknecht, 2011). The American Indian/Alaskan Native populations are spread among approximately 560 federally recognized communities in 34 states and multiple urban areas (Centers for Disease Control and Prevention, 2009). The findings of this dissertation study did not show excess burden of influenza A virus in rural aquatic habits. However, further studies in these areas are

warranted. Excess burden of influenza A virus in rural communities may initiate government direction to health-care providers of American Indian/Alaskan Natives to promote greater awareness of influenza A virus. An influenza awareness campaign may include: the potential severity of influenza A virus infection and current recommendations regarding the timely use of antiviral medications. At the time of this writing the current WHO phase of pandemic alert for Pandemic (H1N1) 2009 was post-pandemic (World Health Organization, 2013).

Government Responses to Pandemic Flu

Government responses to pandemic influenza outbreaks have been both reactionary and tactile. Government responses have been the mass culling of wild and domestic bird flocks. In Pennsylvania following the April to October 1983 outbreak of H5N2 avian influenza virus (low-pathogenicity to high-pathogenicity transformation), the depopulation of over 16 million birds from 380 flocks over three states was accomplished (Henzler, et al., 2003). This was followed by a second depopulation of 350,000 birds in December 1985. Henzler, et al. (2003) wrote the aggressive control and eradication efforts of the second depopulation resulted from the detection of H5N2 low pathogenic avian influenza virus in 10-week-old rosters associated to the New York live-bird markets and examined at the Pennsylvania State University Animal Diagnostic Laboratory.

In May 2005, HPAI H5N1 virus caused an outbreak in migratory waterfowl in Qinghai Lake, western China (Tang, et al., 2009). Tang, et al. (2009) wrote that hundreds of millions of chickens and ducks have been depopulated to control the global spread.

However, HPAI H5N1 outbreaks have been reported in at least 60 countries (Tang, et al., 2009). Wibawa, et al. (2011) wrote that following the 1997 H5N1 outbreaks in the Hunan Province of China, Indonesia reported the majority of H5N1 outbreaks worldwide, with 31 of 33 provinces in the country affected and more than 11 million chicken deaths or depopulated. In 1997, a subtype of H5N1 avian influenza virus outbreak occurred in Hong Kong infecting 18 residents and killing 6 (Laver & Garman, 2001). Laver & Garman (2001) wrote that even though the highly lethal H5N1 avian influenza virus did not effectively spread from person to person, culling of all chickens in Hong Kong effectively stopped the epidemic.

On March 29, 2013, an avian influenza A virus (H7N9), not previously reported in humans, was confirmed by the Chinese Center for Disease Control and Prevention (Centers for Disease Control and Prevention, 2013). Centers for Disease Control and Prevention (2013) reported, as of April 29, 2013, China reported 126 confirmed H7N9 humans cases of which 24 (19%) died. In reaction to contain the outbreak, China's Ministry of Agriculture Officials depopulated some 98,000 poultry and subsequently stopped the sale of live birds as city markets (Cohen, 2013). Other government responses to control the spread of pandemic influenza A virus outbreaks have been less extreme.

Government responses to pandemic influenza outbreaks include the *WHO guidelines for pandemic preparedness and response in non-health sector* and the *HHS Pandemic Influenza Plan (November 2005)*. The "*WHO guidelines for pandemic preparedness and response in non-health sector"* plan involves various sectors of society including: health and non-health sectors, communities, families, and individuals (World

Health Organization, 2009). The "*HHS Pandemic Influenza Plan (November 2005)"* plan further addresses issues outlined in the "*WHO guidelines for pandemic preparedness and response in non-health sector"* plan. These issues include: the provision of anti-viral medications, social distancing, and treatment of secondary infections (U.S. Department of Health and Human Services, 2005). The Shive (2011a) manuscript is a well-detailed framework of public health response to pandemic influenza outbreaks that further complements the WHO and HHS pandemic influenza plans.

Shive (2011a) proposed public health response to influenza virus to be based on principles of infection control as applied to general and specific populations. Primary prevention of influenza infection is the application of universal precautions in the management of influenza-like-illness (Shive, 2011a). Shive contends that secondary prevention of influenza epidemic is early detection and identification. Methods for early detection and identification influenza viruses include: rapid antigen tests, reverse-transcription polymerase chain reaction (RT-PCR), viral isolation, immunofluorescence assays (IFA) and serology (Shive, 2011a). Shive predicates that tertiary prevention of influenza epidemic is the treatment of influenza using anti-viral medications as amantadine, rimantadine, peraivir, oseltamivir, and zanamivir. Additionally, Shive asserted that earlier in the course of influenza infection anti-viral medications are administered, the more effective it is for the patient. This dissertation study to survey artificial aquatic environments is supported by the work of Shive and the WHO and HHS pandemic influenza plans.

Shive and Kanekar (2011) contended one of the emerging areas concerning pandemic influenza is "risk perception". A purpose of this dissertation study is to bring greater public awareness to the human-animal interface of influenza A virus at artificial recirculating water ponds. This investigation may lessen the pyschological threat of unknown risk by bringing greater awareness of how influenza is introduced into the human population. Section 3 of the "*WHO guidelines for pandemic preparedness and response in non-health sector"* plan described current surveillance assessment and included recommendations for improvement for pre-pandemic and during pandemic events (Shive & Kanekar, 2011). Part 2 of the "*HHS Pandemic Influenza Plan (November 2005)"* plan also covered pandemic influenza surveillance. The findings of this dissertation study can be used as baseline measures for the burden of influenza A virus in aquatic environments in urban and rural community settings. The baseline measures may be of importance to future influenza pandemic surveillance efforts as described in the WHO and HHS pandemic influenza plans. Further, this investigation may detect influenza A viruses originating in southern China.

Origin of Influenza (China)

The literature suggests China, with its abundance of live poultry and swine markets, maybe the epicenter of novel influenza viruses. Wan, et al. (2005) proclaimed that southern China has been shown to be the avian influenza virus pool for flu outbreaks in history including H2N2 (1957), H3N2 (1968), H5N1 (1997 & 2003), and H9N2 (1999). Webster et al. (1992) noted historical records and the appearance of the Asian, Hong Kong, and Russian pandemic strains of influenza virus in China suggest the

majority of pandemics of human influenza since about 1850 have originated in China. Perez-Ramirez et al. (2011) described how large parts of Asia support high densities of humans, backyard poultry (ducks, geese, and chickens), pigs, and wild birds. These high density areas provide opportunities for close interaction between influenza reservoir animals and create a unique environment for influenza evolution (Perez-Ramirez et al., 2011). These unique environments may be considered an expansion of the mixing vessel theory; whereas these high density areas may be identified as "mixing vessel environments".

Using historical records, Langford (2005) defended the argument the 1918 – 1919 Spanish influenza pandemic originated in China and global spread resulted from the transportation of Chinese laborers worldwide. Webster et al. (1992) notably suggested the 1918 – 1919 Spanish influenza pandemic originated in military camps in Kansas and was taken to Europe by U.S. Troops in 1918. Nearly ninety years following the pandemic 1918 Spanish flu, there is evidence suggestive that novel influenza outbreaks originate in China.

In May 2013, the Centers for Disease Control and Prevention (2013) reported the March 2013 outbreak of H7N9 was confirmed in eight contiguous provinces in eastern China (Anhui, Fujian, Henan, Hunan, Jiangsu, Jiangxi, Shandong, and Zhejiang), two municipalities (Beijing and Shanghai), and Taiwan. Further, Centers for Disease Control and Prevention reported that no other human cases of H7N9 virus infection have been detected outside of China, including the United States. The early reports of the March 2013 outbreak of avian influenza A (H7N9) suggested China to be the geographical

origin. However, Chinese news media has not shown to be supportive that China is the epicenter of influenza viruses.

In the retrospective study by Heffernan, Misturelli, & Thomson (2011), researchers examined Chinese news media reporting of avian influenza outbreaks. The results suggested the Chinese government "down played" the events leading to under-reporting/misrepresenting of the disease while depiction of a strong and efficient 'China' that was a global leader in the fight against the disease. The literature support of the hypothesis that China is the epicenter for influenza viruses underscores this dissertation study.

Ito et al. (1995) asserted waterfowl as ducks and geese in the northern hemisphere migrate between Alaska, Canada, and Siberia resulting in the transportation and shedding of influenza virus. Additionally, a majority of migratory ducks in Asia are from Siberia (Ito et al., 1995). An avian influenza A virus infected migratory bird could potentially carry the virus to the migratory bird breeding grounds of Siberia from where transmission to another migratory bird could possible occur. This second bird could potentially carry the virus to the breeding grounds of Alaska or Canada where transmission to another migratory bird could possible occur. This third bird could potentially carry the virus South from the breeding grounds of Alaska or Canada to Central and South America. Therefore, it is possible for a novel avian influenza A virus originating in China to be detected in waterfowl or wetlands along the eastern Pacific flyway region. The contamination of aquatic habitats with influenza A viruses along the migratory flyways may result in influenza A virus infection of other animals. Other animals may include

swine. Swine are susceptible to avian and human influenza A viruses which is the foundation of the Mixing Vessel Theory.

Scholtissek et al. (1985) Mixing Vessel Theory

Mixing vessel theory consists of three parts: (i) swine are susceptible to avian and human influenza A viruses, (ii) reassortment of swine/avian/human viruses occurs in the pig, and (iii) pigs can transmit reassortant influenza viruses to people (Ma et al., 2009 p. 330). Ma et al. (2009) investigated the validity of the three parts of Mixing Vessel Theory using published literature. Ma et al. concluded in support of Mixing Vessel Theory and swine as a potential mixing vessel for influenza viruses. Additionally, the scientific literature supports Mixing Vessel Theory not to be limited only to swine; domestic fowl are also considered to be intermediate hosts for virus reassortment (Ma et al., 2009).

Bergsmedh, Ehnfors, Spetz, and Holmgren (2007) investigated if the theory of horizontal gene transfer applied to viruses, including influenza viral strains. The researchers applied advanced biotechnology techniques including cell culture and fluorescence in-situ hybridization techniques. The proposed theoretical view is phagocytosis of apoptotic cells (the clean-up of dead cell by macrophages) is a method for RNA transfer between cells, and hence, larger organisms. The researchers were successful in replicating this process in-vitro (Bergsmedh, Ehnfors, Spetz, & Holmgren, 2007). However, in-vivo RNA transfer is still hypothetical. Bergsmedh et al. (2007) contributed to the theoretical view that dabbling ducks can introduce and host influenza A viruses through the process of ingestion and defecation.

Eastel (1988) focused on molecular clock hypothesis as it applies to placental mammals. The research by Easteal computed phylogenetic analysis to genetic data from GenBank. These studies contribute a temporal comparison of DNA evolutionary rates to RNA viral (influenza viruses) evolutionary rates.

Louis Pasteur's Germ Theory

Pasteur's Germ Theory identifies germs of microscopic organisms as the cause of disease, and the theory of spontaneous generation is chimerical (Pasteur et al., 1878). Underlining the theoretical framework for this study were Pasteur's (1857-1858) Germ Theory and Koch's (1890) Postulates as adapted for viruses.

Koch's Postulates Applied to Viruses

Koch's Postulates as adapted for viruses implies certain conditions-not all of Koch's Postulates-have to be met before a specific relation of a virus to a disease is established. The conditions are: (i) a specific virus must be found associated with a disease with a degree of regularity, and (ii) the virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation (Rivers, 1937).

Theories in the Literature

Applications of Pasteur's Germ Theory and Koch's Postulates as adapted for viruses are present in the literature. As previously noted in this chapter, animal-to-human transmission of influenza virus is considered "primary" transmission and human-to-human transmission of influenza virus is considered "secondary" transmission (Lloyd-Smith, et al., 2009). The literature recognizes personal hygiene and vaccination as the

most important and effective methods for controlling for transmission of influenza viruses (McClure et al., 2011). Mao and Yang (2012) posit human networks, infectious diseases, and human preventive behavior are intrinsically inter-related (Mao & Yang, 2012). Personal hygiene is an extension of human preventive behavior for controlling the spread and secondary transmission of influenza viruses. Avoidance of animal-human contact with dead or sick birds is the best prevention for preventing H5N1 primary transmission and infection (Centers for Disease Control and Prevention, 2010).

Vaccine and vaccination is another topic where Pasteur's Germ Theory and Koch's Postulates as adapted for viruses are present in the literature. Vaccination is an accepted method for reducing transmission and infection from germs. However, vaccine development is a process which can take months to years to develop and produce ample supply for national distribution (Hessel, 2011). Additionally, vaccine production and availability cannot guarantee full compliance by all individuals. A recent study found that 80% of pregnant women believed flu vaccines could cause birth defects and only 50% of the women were aware of the national recommendations for vaccination during pregnancy (Durica et al., 2011). These findings by Durica et al. (2011) bring to light the challenges of secondary transmission monitoring and control of influenza viruses.

A predominance of the monitoring and control of HPAI H5N1 has been accomplished by cloacal swab sampling of migratory birds. This is a process of monitoring for morbidity and mortality events. Morbidity and mortality events are consistent with Pasteur's Germ Theory and Koch's Postulates as adapted for viruses. This process is labor intensive, and morbidity and mortality trending tends to strongly bias

towards birds which are susceptible to the virus (Farnsworth, et al., 2011). Migratory birds that are asymptomatic can spread the virus across long distances including national borders (Gaidet, et al., 2010).

Attempts to control the spread of avian influenza viruses are consistent with Pasteur's Germ Theory and Koch's Postulates as adapted for viruses. Control measures for outbreaks of avian influenza viruses have included the depopulation of commercial birds and flocks. The depopulation of bird flocks is an attempt to control primary and secondary transmission of avian influenza viruses. During the October 1984 HPAI H5N2 outbreak in Pennsylvania, more than 17 million birds were depopulated (Okonkwo & Lupankwa, 2011). A recent study estimated between 2004 to 2008, nearly 29 million birds had been culled in China in an effort to control HPAI H5N1 (Oyana, McGoy, & Dai, 2011). However, by not culling flocks of birds, evolutionary pathways may develop poultry lineages that are HPAI H5N1 resistant.

A different approach to monitoring and controlling for HPAI H5N1 human cases would be environmental water monitoring for HPAI H5N1 viruses. The literature supports migratory waterfowl as the natural reservoir for influenza A viruses (Webster et al., 1992). Additionally, the literature supports a fecal-oral transmission between waterfowl of influenza A viruses (Franklin et al., 2011). Geographical water analysis-where waterfowl are found-for influenza viruses may detect HPAI H5N1 strains. This environmental monitoring approach could provide public health officials with geographic specific data for influenza intervention and response efforts. As aforementioned, by not

culling flocks of birds, evolutionary pathways may develop poultry lineages that are HPAI H5N1 resistant.

Migratory Waterfowl (Vector for Global Distribution)

Austin and Hinshaw (1984) investigated feral duck species as a source of transmission of influenza A virus and paramyxoviruses. The researchers collected swab samples from tracheal and cloacae from different species of feral ducks (Austin & Hinshaw, 1984). Austin and Hinshaw and were able to identify several viral strains of influenza A virus. Additionally, Austin and Hinshaw asserted the alterations of influenza viral strains occur due to antigenic drift at point mutations and antigenic shift is caused by genetic reassortment. The authors contended it was possible these alterations occurred in the intestinal tract of ducks (Austin & Hinshaw, 1984). This article provides support to the hypothesis that migratory ducks are a source point for influenza viruses. Therefore, surveillance of healthy ducks and the aquatic environment they frequent may be of significant interest to monitoring and controlling influenza A viruses.

Gaidet et al. (2010) focused on the long-range migratory patterns of waterfowl to explain the global dispersion of highly pathogenic avian influenza H5N1 virus (HPAI H5N1). Satellite telemetry was used to study the movements of migratory waterfowl over Asia, Europe, and Africa. The authors concluded, on average, a migratory bird would only have 5-15 days per year of infection to disperse HPAI H5N1 (Gaidet, et al., 2010). Thus, intercontinental virus dispersion requires relay transmission between series of successfully infected migratory waterfowl. Additionally, only asymptomatic birds could successfully disperse HPAI H5N1 (Gaidet, et al., 2010). This study supports the theory

the seasonality of influenza outbreaks can be explained, in part, by movements of migratory birds. However, population studies of migratory birds are not a reliable influenza disease surveillance tool.

Hierarchical modeling can be used to estimate bird populations. Link and Sauer (2002) focused on hierarchical modeling for estimating Cerulean Warblers populations using North American Bird Breeding Survey data. The work by Link and Sauer is critical of the validity of North American Bird Breeding Survey data. The authors asserted that North American Bird Breeding Survey data is not well suited for monitoring the Cerulean Warblers (Link & Sauer, 2002). Reasoning for this critical judgment is the flight patterns of the Cerulean Warblers are not compatible with the observation methods of North American Bird Breeding Survey. This article provides opinion about the application and validity of the population trends of the North American Bird Breeding Survey data. Additionally, this article rules-out the association of migratory bird population data to seasonal influenza outbreaks. However, the studies of migratory waterfowl as a host is still of great importance in influenza A virus surveillance.

Mehrabanpour et al. (2012) focused on cloacal swabs and samples of bird droppings from the wetland regions of Boushehr, Iran. Samples were analyzed for influenza viral strains using RT-PCR methodologies. These studies and others have identified influenza viral subtypes in various waterfowl breeds (Ferro, et al., 2010; Mehrabanpour, et al., 2012). These studies support the hypothesis that migratory waterfowl play a role in the natural influenza virus reservoir(s) and dispersal. Similar studies have been conducted in the United States.

Breeding Grounds as Hotspots

Ferro et al. (2010) focused on the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas Coast. Cloacal swabs were analyzed for influenza A viruses using RT-PCR technique. Over a three year period, the researchers were able to collect samples from 5,363 birds. Data analysis showed a prominence of specific sub-strains of influenza and also identified Blue-winged Teals and Green-winged Teals as carriers of several influenza viral subtypes (Ferro, et al., 2010). These studies provide additional evidence that influenza viruses can be identified among health migratory waterfowl.

Water Analysis

Water analysis has been an important scientific approach to understanding the epidemiological triangle model of influenza viral diseases. The epidemiological triangle model of disease causation is the relationship of transmission, agent, and host. In the early study by Cumming (1919), a case-control approach was used to better understand the transmission and infectivity of the causitive agent of influenza-pneumonia. The influenza-pneumonia organism was suspected to be sputum-borne and transmission occurred through inanimate objects by the hand and mouth route (Cumming, 1919). In the Cumming study, the agent was the influenza-pneumonia organism, the route of transmission was contaminated eating utensils, and aquatic dishwashing was the environment.

The case-control approach of the Cumming (1919) study compared influenza-pneumonia rates in populations of public institutions: aged, State hospitals, State prisons,

Federal prisons, Tuberculosis sanatoriums, Feeble-minded, and Children where mechanical dishwashing was used and not used (Cumming, 1919). Cumming hypothesized that scalding hot water used in mechanical dishwashing disinfected the eating utensils and dishware, whereas luke warm water would not. The data for the Cumming study was culminated from questionaires returned from 370 public institutions. The institutuional population for this study is [n = 252,186] (Cumming, 1919). The data analysis showed an inverse relationship between scalding hot water use for dishwashing and influenza-pneumonia illness. Recent studies by others have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen et al., 2010; Webster et al., 1992).

Webster et al. (1992) asserted waterborne transmission of influenza viruses occurs due to the viral shedding in the fecal material of waterfowl (Anseriforms) and shorebirds (Charadriiformes). VanDalen et al. (2010) investigated waterborne transmission of influenza A H4N6 in mallards (*Anas platyyrhychos*) in a controlled laboratory environment. The finding of this research showed that influenza A H4N6 can be successfully transferred to naive ducks via influenza contaminated water (VanDalen et al., 2010). Additionally, VanDalen et al. were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Thus, it is possible that influenza viruses can be transfered between organisms via open water-sources as wetlands, lakes, and ponds. Other researchers have investigated if influenza A viruses can be detected in these contaminated aquatic habitats (Lang et al., 2008; Stallknecht & Brown, 2009; Zhang et al., 2006).

Lang, et al. (2008) collected and analyzed sediment samples from three ponds in the Creamer's Field Migratory Waterfowl Refuge, Alaska, a location used by a wide variety of migratory waterfowl. The sediment samples were collected using a time-series approach and analyzed for influenza A virus RNA using reverse transcription-polymerase chain reaction (RT-PCR) methodologies (Lang et al., 2008). Lang, et al. sequenced the sediment samples for influenza A virus M gene, H3 gene, H8 gene, H11 gene, and H12 gene. The sediment samples from the three ponds were positive for various combinations of influenza A virus M, H3, H8, H11, and H12 genes (Lang et al., 2008).

Lang, et al. (2008) asserted that pond sediment sampling and analysis is a powerful technique to study the diversity of influenza viruses present in an enviromental location, and therefore present in birds that utilize the location. Additionally, the authors asserted pond sediment sampling is advantagous to direct sampling of birds because direct sampling of birds is limited to times when birds can be captured efficiently and effectively (Lang et al., 2008). Thus, the viral samples collected from direct bird sampling may be temporally limited. Another method of environmental sampling for influenza A viruses, similar to the approach in the Lang, et al. study, is the collection and analysis of samples of frozen lake ice where migratory birds may refuge or over winter.

Zhang et al. (2006) collected and analyzed samples of ice or water from three northeastern Siberian lakes in the Koluma River region. The samples were analyzed for the presence of influenza A virus using reverse transcription-polymerase chain reaction (RT-PCR) methodologies (Zhang et al., 2006). Zhang et al. asserted these lakes are covered by ice for more than 6 months a year and are frequented by Chinese-Siberian

axis populations of migratory waterfowl, some of which travel to North America and others that travel as far as southern Asia, Europe, and Africa.

Three notable findings in the Zhang et al. (2006) study are: (i) the highest frequencies of detection of influenza A virus RNAs are in the lakes with the highest concentrations of migratory waterfowl, (ii) influenza A virus RNA is preserved in higher concentrations in lake ice than in lake water, and (iii) the H1 gene population in the lakes is genetically heterogeneous. Additionally, the authors of the Zhang et al. study posit ice may act as a reservoir for influenza A viruses, preserving them for later release and infection of animals, including migratory waterfowl and humans. If lake ice is a natural reservoir for influenza A viruses, the Zhang et al. study may provide valuable insight to explaining the phenomena of influenza A viral strains emerging, disappearing, and then re-emerging over time. However in the Worobey (2008) study, the findings by Zhang et al. are challenged.

Worobey (2008) asserted the influenza A virus hemagglutinin gene sequences detected in the Siberian lake ice by Zhang et al. (2006) study originated from a laboratory reference strain derived from the earliest human influenza A virus isolate, WS/33. If the research by Worobey is correct, the Siberian “ice-viruses” identified in the Zhang et al. study are contaminants and there is no credible evidence that environmental ice acts as biologically revalent reservior for influenza viruses (Worobey, 2008). Other researchers have studied influenza A viruses in surface water samples to investigate the perpetuation between waterfowl and water-contamination (Hinshaw et al., 1980; Ito et al., 1995),

while others have researched the infectivity and transmission of influenza A viruses in the aquatic environment (Brown et al., 2009; Halvorson et al., 1985).

Hinshaw et al. (1980) collected samples from waterfowl, unconcentrated lake water, and feces from lake shores near Vermillion, Alberta, Canada. These samples were used to isolate influenza viruses and to investigate whether influenza viruses continually circulate or whether the same or different strains are present from year to year (Hinshaw et al., 1980). Hinshaw et al. asserted this longitudinal study of feral ducks would provide data on the size of the gene pool of influenza A viruses in nature and the number of viruses antigenically related to human strains present in avian species. Additionally, the authors concluded that this study shows that (i) many different ortho and paramyxoviruses circulate every year in various species of apparently healthy ducks in the marshalling areas of Vermillion; (ii) the antigenic subtypes and the frequency of the viruses change from year to year; (iii) antigenic drift occurs in the avian viruses; and (iv) viruses antigenically related to previous human strains continue to circulate in the ducks (Hinshaw et al., 1980). Ito et al. (1995) conducted a similar investigation in Alaska.

Ito et al. (1995) collected and analyzed influenza virus isolates from the fecal samples of dabbling ducks and also from the lake water used by migratory waterfowl. The purpose of this study was to investigate the theory of water-borne transmission of influenza virus (Webster et al., 1992) as a mechanism of year-to-year perpetuation of the viruses in lakes where migratory waterfowl may breed (Ito et al., 1995). The researchers of the Ito et al. study collected fecal and water samples each July from 1991 to 1994. The fecal samples were collected from 15 different points in Alaska, and 81 water samples

were collected from 11 different ponds and lakes where ducks and geese were nesting (Ito et al., 1995). The authors of the Ito et al. research asserted the findings of viral isolation from multiple lake and pond samples of the same subtypes over the course of this longitudinal study support the hypothesis that influenza viruses are maintained in waterfowl by a water-borne transmission mechanism. Additionally, the researchers were able to show the viruses remained viable in the lake water after most ducks left for migration South (Ito et al., 1995). Further analysis of influenza virus viability and infectivity in water was investigated by Brown, Goekjian, Poulson, Valeika, and Stallknecht, (2009).

Unlike the natural environmental studies by Hinshaw et al. (1980), and Ito et al. (1995), the investigation by Brown et al. (2009) was performed in a laboratory setting. The researchers of the Brown et al. study sought to evaluate abiotic environmental factors affecting avian A influenza virus persistence in aquatic habitats. Specifically, the researchers of the Brown et al. study investigated the duration of infectivity for avian influenza viruses representing a diversity of HA subtypes (H1 – H12) isolated from anseriforms and charadriiforms over a range of pH, salinity, and temperature conditions that are common in aquatic habitat environments utilized by these wild aquatic birds. For each virus/pH/salinity/temperature trial, infectivity assay endpoints were measured at 100% destruction of a monolayer of Madin Darby canine kidney cells (Brown et al., 2009).

Brown et al. (2009) summarized that infectivity of most avian influenza A virus strains persisted longest at slightly basic water conditions (for both 17°C and 28°C), pH

values 7.4 – 8.2; that increasing water temperatures had a negative effect on the persistence of all avian influenza A viruses examined; and increasing salinity also had a negative effect of avian influenza A viral persistence in water (for both 17°C and 28°C). Brown et al. further asserted the findings of this study indicate the pH, temperature, and salinity-at levels normally encountered in nature-can impact the ablity of avian influenza A viruses to remain infective in water. Early research by others directly investigated the infectivity and water-borne transmission of of avian influenza A viruses using sentinel ducks and sentinel turkeys (Halvorson et al., 1985).

In the four year study by Halvorson et al. (1985), flocks of sentinel ducks (negative for avian influenza) were allowed to comingle with released ducks and wild ducks in specially constructed pens located at selected marshes. During the third and fourth years of this study, sentinel turkeys were introduced into pens at two locations where the turkeys could comingle with ducks, share feed sources, and drink pond water (Halvorson et al., 1985). The data analysis of the Halvorson et al. investigation showed a seasonal pattern to the detection of influenza A virus isolations in both the sentinel ducks, as well as, the sentinel turkeys.

Interestingly, there was a six to eight week lag period from the onset of infection in sentinel ducks and turkey flocks (Halvorson et al., 1985). The authors of the Halvorson et al. (1985) study suggested that this six to eight week lag of the onset of infection in turkey flocks maybe due to several possibilities: waterfowl activity and duck movements; other vectors or vehicles such as transportation vehicles to move turkey flocks; abiotic environment conditions such as temperature; ambient surface water temperature;

groundwater contamination; and viral adaptation. Halvorson et al. provides support for the theory of water-borne transmission of avian influenza A viruses among wild waterfowl and domestic fowl, even though the research only identified certain duck-derived isolates are capable of infecting domestic turkeys. Other researchers have investigated animal-to-animal and human-to-animal transmission of influenza outside of water-borne transmission to fill gaps in the knowledge of the molecular characterization of influenza A viruses.

Influenza Virus Molecular Structure

Gibson, Bowman, and Connor (1919) investigated sputum collected from patients infected with influenza in early stages of the disease. In this first phase of the investigation, emusions of filtered and unfiltered sputum preparations were inoculated to baboons, Macacus rhesus monkeys, rabiits, guinea pigs, and mice (Gibson, Bowman, & Connor, 1919). The researchers of the Gibson et al. study reported that by inoculation of sputum, both filtered and unfiltered, from cases of influenza, successfully produced, in all animals, lesions that appeared to be similar to those seen in human cases of the disease. The second phase of the Gibson et al. research continued inoculation-animal experiments using blood from human cases of influenza infection. The outcomes of human blood inoculation of animals was a mix of positive and negative results (Gibson et al., 1919).

The third phase of the Gibson et al. (1919) research investigated the passage of the virus from animal to animal. The findings of this phase of the research suggested that there may be an increase in the virulence when the virus is passed through one animal to another (Gibson et al., 1919). The fourth phase of the Gibson et al. research investigated

inoculation of animals using cultures. The influenza cultures were prepared using selected ascites fluid in the Noguchi method (Gibson et al., 1919). The prepared Noguchi culture solutions were inoculated in to animals and post-mortem examination followed (Gibson et al., 1919). Gibson et al. concluded the cultures when inoculated into animals produced typical "experimental influenza" lesions. Reproduction and isolation of the influenza virus by animal-culture was further investigated by Bashford (1919) and (Wilson, 1919).

Bashford (1919) investigated animals inoculated with pure culture and sub-cultures to investigate the reproduction of influenza disease. Inoculation from first generation animals to second generation animal to third generation animals was done using blood, bile, or urine (Bashford, 1919). Bashford concluded the positive findings of inoculation using pure and sub-cultures satisfied the requirements of Koch's Law. Wilson (1919) investigated cultures of certain filter-passing organisms, including influenza, using the Noguchi method. Wilson reported positive growth in culture and described influenza organism as a "minute rounded or oval coccus-like body of very definite outline and of uniform appearance…varies from 0.15 μ to 0.5 μ…" (p. 603). Shortly after the work by Wilson was published in *The British Medical Journal*; Arkwright (1919) challenged the findings and conclusions by Bashford (1919) and (Wilson, 1919).

Arkwright (1919) proclaimed the filter-passing influenza organisms isolated and studies by Bashford (1919) and Wilson (1919) were bacterial contaminants. Bashford and Wilson reported the macroscopic appearance of the Noguchi culture tubes as having a high degree of turbidity. Arkwright argued these highly-turbid tubes in question could

have been demonstrated to contain bacterial growth by readily available methods as Giemsa, Gram and fuchsin, and by culture in agar and in broth. As is commonly known, viruses cannot be seen macroscopically or microscopically. Thus, the argument presented by Arkwright that Bashford and Wilson isolated bacterial organisms, *not influenza viruses* is likely valid.

Influenza viruses are named *Orthomyxoviridae* (*ortho* meaning "standard, correct" and *myxo* meaning "mucus") because of their ability to bind mucus and to differentiate them from *Paramyxoviridae*, another group of negative-sense RNA viruses (Poulson, 2011). There are five genera of *Orthomyxoviridae*: influenza A virus, influenza B virus, influenza C virus, thogoto-virus, and isa-virus (Taubenberger & Kash, 2010). The work by Poulson (2011) and Taubenberger and Kash (2010) provides current detailed understanding of the molecular structure of influenza A virus. Wali and Music (2011) summarized current understanding of influenza viron structure. As is currently accepted, the influenza viruses envelop contains two virus-coded glycoproteins spikes, the hemaggultinin (HA) and neuraminidase (NA) proteins which are the primary antigens in the host response to influenza virus inflection (Wali & Music, 2011).

Influenza A subtypes are classified by the antigenic groups HA and NA. At the time of this writing, the World Health Organization recognizes 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes (Poulson, 2011). There are eight discrete single-stranded RNA gene segments found in influenza A and B viruses (Lebarbenchon & Stallknecht, 2011). These eight single-stranded RNA gene sequences are: PB2, PB1, PA, HA, NP, NA, M, and NS (Taubenberger & Kash, 2010).

PB2 Polymerase

Basic Polymerase Protein 2 (PB2) RNA polymerase subunit is encoded in segment 1 of the influenza A virus (Cheung & Poon, 2007). Cheung and Poon (2007) stated that PB2 is required for viral transcription and replication. PB2 polymerase functions during initiation of influenza A viral mRNA transcription as the protein which recognizes and binds the 5' capI structures of host cell mRNAs for use as viral mRNA transcription primers (Webster et al., 1992). Cheung and Poon suggested PB2 has endonuclease activity and uses host mRNA to generate cap primers for viral RNA synthesis. Apart from the biological functions of PB1, the literature suggests PB1 maybe a major determinant in controlling the pathogenecity of the influenza A virus (Cheung & Poon, 2007).

PB1 Polymerase

Basic Polymerase Protein 1 (PB1) RNA polymerase subunit is encoded in segment 2 of the influenza A virus (Cheung & Poon, 2007). Cheung and Poon (2007) suggested that PB1, itself, is an RNA polymerase. PB1 polymerase functions in the RNA polymerase complex as the protein responsible for elogation of the primed nascent viral mRNA and also as elongation protein for template RNA and vRNA synthesis (Webster et al., 1992). Further, the PB1 subunit is involved in the assembly of three polymerase protein subunits and the catalytic function of RNA polymerization (Cheung & Poon, 2007).

PA Polymerase

Acidic Polymerase Protein (PA) is encoded in segment 3 of the influenza A virus (Cheung & Poon, 2007). PA polymerase localizes in the infected cell nucleus and functions as part of the RNA-dependent RNA polymerase complex in conjunction with PB1 and PB2, however, its role in viral RNA synthesis is not well understood (Webster et al., 1992). Cheung and Poon (2007) identified several biological functions of PA polymerase: nuclear transportation signals required for effcient nuclear accumulation of the PB1 subunits, endonucleolytic cleavage of capped RNAs, and the initiation of proteolysis in infected cells. However, the complete functions of PA are still not well understood (Cheung & Poon, 2007).

Hemagglutination

Hemaggultination (HA) is encoded in segment 4 of the influenza A virus (Cheung & Poon, 2007). The HA protein is an integral member protein and a primary surface antigen of the influenza viron (Webster et al., 1992). At the time of this writing, there were 16 HA subtypes recognized by the WHO (Poulson, 2011). The HA protein is responsible for the binding for viral particles to sialic acid-containing receptors on host cells and is a major target for humoral neutralizing antibodies (Cheung & Poon, 2007). According to Taubenberger and Kash (2010), HA protein recognizes sialic acid bound to underlying sugars on the tips of host cell glycoproteins. This recognition leads to HA-receptor binding followed by internalization of the virus, and where the acidic pH of the endosomal compartment causes a conformational change of HA resulting in mediating fusion of the viral and endosomal membranes, thus allowing release of viral

ribonucleoprotein complexes into the host cytoplasm (Cheung & Poon, 2007; Taubenberger & Kash, 2010). Prior to internalization, the HA protein is synthesized as a precursor polypeptide, HA_0, which is post-translationally cleaved in to two subunits: HA_1 and HA_2 (Cheung & Poon, 2007). Cheung and Poon stated the cleavage of the HA_0 is a prerequisite for viral infectivity. Additionally, Cheung and Poon (2007) further asserted there is a direct relationship between the quantity of basic amino acids near the HA protein cleavage site and the virulence of highly pathogenic viruses.

Nulceoprotein

Nucleoprotein (NP) is encoded in segment 5 of the influenza A virus (Cheung & Poon, 2007). Nucleoprotein is transported into the infected cell nucleus where it binds to and encapsidates viral RNA (Webster et al., 1992). Taubenberger and Kash (2010) stated each influenza A virus RNA segment is encapsidated by NP. Cheung and Poon (2007) stated NP is one of the essential components for transcription and replication of influenza A virus RNA. However, dissociation of the NP from the RNA template is not thought to be required for viral transcription and replication (Cheung & Poon, 2007). Webster et al. (1992) suggested nucleoprotein may play a role in the switching of viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis.

Neuraminidase

Neuraminidase (NA) is encoded in segment 6 of the influenza A virus (Cheung & Poon, 2007). The NA protein is an integral membrane glycoprotein and a second major surface antigen of the influenza viron (Webster et al., 1992). Similar to HA, NA is a major antigenic target for humoral neutralizing antibodies (Taubenberger & Kash, 2010).

At the time of this writing, there were 9 NA subtypes recognized by the WHO (Poulson, 2011). Taubenberger and Kash (2010) identified NA as a type II integral membrane glycoprotein with sialidase enzymatic activity required for cleavage of both host cell sialic acids, thus allowing release of newly produced virons, and sialic acids on viral glycoproteins to prevent aggregation of nacent viral particles. Cheung and Poon (2007) suggested the glycosylation of cell surface NA might be an important determinant of the neurovirulence of influenza viruses. Even though the literature contains a constellation of studies investigating NA, the function of NA activity in the influenza life cycle is not fully understood (Cheung & Poon, 2007).

M1 Protein

Matrix Protein 1 (M1) is encoded in segment 7 of the influenza A virus (Cheung & Poon, 2007). M1 protein is the most abundant protein in the influenza A virus viron (Webster et al., 1992). Webster et al. (1992) explained the colinear transcription of segment 7 produces mRNA for the matrix protein. It is the matrix protein that forms a shell surrounding the virion nucleocapids underneath the viron envelope (Webster et al., 1992).

The shell formed by the matrix protein interacts with cytoplasmic domains of the surface glycoproteins and also with the viral ribonucleoprotein (Taubenberger & Kash, 2010). Cheung and Poon (2007) suggested that M1 protein and vRNA together promote the self-assembly of the influenza A virus NP into a typical quanternary helical structure of viral ribonucleoprotein (vRNP) complexes. Further, the interaction of NP protein with vRNA and M1 in a system devoid of other viral proteins may lead to translocation of

vRNP form the nucleus to the cytoplasm (Cheung & Poon, 2007). Cheung and Poon suggested previously reported studies showing that M1 protein synthesized at high temperatures (41°C) is unable to interact with vRNP and the transfer of vRNP into the cytoplasm is blocked, thus the association between M1 and vRNP is essential for the nuclear export of vRNP. This assertion by Cheung and Poon may provide a mechanism for inhibiting the successful replication of influenza A viruses using a heat-shock treatment.

M2 Protein

Matrix Protein 2 (M2) is encoded in segment 7 of the influenz A virus (Cheung & Poon, 2007). M2 protein is an integral membrane protein (Webster et al., 1992). Webster et al. (1992) suggested the membrane-spanning domain of the M2 protein also serves as a signal for transport to the cell surface. Cheung and Poon (2007) supported this suggestion by Webster et al. by noting the M2 tetramer has ion channel activity for pH regulation. The ion channel activity of M2 is reported to maintain a high pH in the Golgi vessicles so as to stabilize the native conformation of newly synthesized HA during intercellular transport for viral assembly.

Nonstructural NS1 and NS2 Proteins

Nonstructural Protein 1 (NS1) and Nonstructural Protein 2 (NS2) are encoded in segment 8 of the influenza A virus (Cheung & Poon, 2007). Webster et al. (1992) asserted that NS1 and NS2 are abundant in the infected cells; NS1 primarily in the nucleus, and NS2 primarily in the cytoplasm. It is known both NS1 and NS2 play a role in viral replication, however, these roles are not well understood (Webster et al., 1992).

NS1 is the only non-structural protein of the influenza A virus (Cheung & Poon, 2007). Taubenberger & Kash (2010) reported NS1 has multiple functional domains and pleiotrophic functions. In early studies, NS2 was believed to be a non-structural protein, however, Cheung and Poon (2007) reported that several recent studies have indicated NS2 is incorporated into viral particles in amounts. Taubenberger and Kash identified NS2 as a nuclear export protein found in virons and facilitating nuclear export of viral RNP complexes.

Antigenic Shift

Reassortment, also known as *Antigenic shift*, are the sudden changes in the amino acids of the RNA sequences of the HA and NA strands (Poulson, 2011). Poulson (2011) identified three mechanisms of antigenic shift: first, genetic reassortment can occur between circulating human influenza A virus and an avian influenza A virus; second, possible direct transmission of an avian or swine influenza virus from birds to humans and their establishment in the human population; and third, the reintroduction of an "old" or "previous strain" into the population (p. 38).

In 1996, HPAI H5N1 viruses caused and epizootic outbreak in southern China. One year later, the HPAI H5N1 an epizootic outbreak occurred in Hong Kong that resulted in 18 human cases and six deaths (Taubenberger & Morens, 2009). The genetic nature of HPAI H5N1 RNA results in a relatively high rate of nucleotide substitutions (Pfeiffer et al., 2011). These nucleotide substitutions result in point mutations, a process known as "antigenic drift" (Aldras, 2011). The literature suggests that antigenic drift is the principle reason why particular influenza strains continue to circulate (Pfeiffer et al.,

2011). Six years after the epizootic outbreak in China, HPAI H5N1 reappeared in epizootic form and spread widely throughout Southeast Asia (Taubenberger & Morens, 2009).

Antigenic shift occurs only in influenza A strains and results in the emergence of completely new subtypes (Wali & Music, 2011). Antigenic shift is a process of genetic reassortment involving the gene segments encoding the HA and/or NA genes (Taubenberger & Kash, 2010). At the time of this writing, there were sixteen HA and nine NA known subtypes, therefore, theoretically there are 144 possible HA-NA subtype combinations possible. Further, there are theoretically 256 (2^8) possible combinations of the eight RNA gene segments from reassortment between two parental viruses (Taubenberger & Kash, 2010).

Antigenic Drift

Wali and Music (2011) and others asserted that antigenic drift is the result of mutation(s) affecting the RNA segments coding for either hemagglutinin or neuraminidase, but more commonly hemagglutinin. These mutations are changes in the amino acid sequences of the antigenic portions of the surface glycoproteins HA and NA (Taubenberger & Kash, 2010). Taubenberger and Kash (2010) and others asserted antigenic drift may produce selective advantages for viral strains by allowing them to evade pre-existing immunity. The term "mutation" is synonymously used to describe "antigenic drift". Poulson (2011) asserted antigenic drift is the result of the lack of a proofreading mechanism and the relatively low fidelity of the RNA-dependent RNA polymerase causing errors to be made during the RNA replication process (p. 38).

Antigenic shift is a gradual process, unlike the sudden and more disruptive process of antigenic shift (Poulson, 2011).

Laboratory Assays

A constellation of influenza virus investigations have been supported by laboratory assays. Some research studies have investigated influenza viruses in the natural environment (Vong, Ly, Mardy, Holl, & Buchy, 2008), while others have approached the topic in the laboratory setting (Faust et al., 2009). The methodology for characterization of influenza viruses has shown similarity across the literature. Overarching methodologies include: virus detection by RT-PCR (Poddar et al., 2002), virus isolation and characterization by allantoic cavities of embryonic eggs (Fouchier, et al., 2005), infectivity studies using MDCK cell line (Brown et al., 2009), nucleotide sequencing of the RNA genome (Yamamoto, et al., 2011), and phylogenic analysis using BLAST available from GenBank (Fouchier, et al., 2005). The majority of influenza samples for laboratory analysis have been cloacal swabs and oropharyngeal swabs from migratory waterfowl (VanDalen et al., 2010). A limited number of studies have collected environmental water samples from migratory waterfowl breeding grounds (Zhang et al., 2011).

RT-PCR

Perez-Ramırez, et al. (2012) investigated influenza A virus detection by real time RT-PCR was conducted on Spanish wetland water samples. The purpose of the Perez-Ramırez, et al. study was to determine the influence of a range of ecological factors (climate conditions, density and diversity of wild birds, water physico-chemical

properties and shelter and food availability) in avian influenza virus dynamics in the Spanish wetlands under field conditions. The researchers in this study used TaqMan RRT-PCR specific for the matrix gen (M gene) in the segment 7 of avian influenza A virus, the Qiagen QIAamp viral RNA extraction for the viral extration, and the Life Technologies-Applied Biosystems one-step RT-PCR kit for amplification (Perez-Ramırez, et al., 2012). The Perez-Ramırez, et al. study is support for the use of real time RT-PCR specific for the matrix gene (M gene) to identify influenza A virus from environmental water samples in this dissertation research.

Evers et al. (2007) investigated the commercial preservative RNAlater (Qiagen) by testing and comparing against the current method of cryo-freezing, and ethanol preservatives for influenza A virus samples. The purpose of this investigation was to determine if using the commercial preservative RNAlater would result in improved RT-PCR amplification over the current sample preservation methods of cryo-freezing or ethanol fixation (Evers et al. 2007). From the findings of this study, the authors asserted the commercial preservative RNAlater held at ambient temperatures might be useful for the identification of influenza A virus in samples collected from infected waterfowl (Evers et al. 2007). The application of the Evers et al. study may provide an alternative surveillance method influenza A virus using commercially available preservative to reduce costs, time, labor, and be more practical in remote areas. The Evers et al. study is support for the use of the commercial preservative RNA later in this dissertation research.

Vong, Ly, Mardy, Holl, and Buchy (2008) invesigated H5N1 influenza A virus detection by real time RT-PCR using viral collection swabs. The purpose of this

investigation was to determine the potential risk of bird-to-human transmission of H5N1 influenza A virus outbreaks among backyard poultry in Cambodia (Vong et al. 2008). The authors of the Vong et al. study concluded viral RNA was frequently present on various enviromental surfaces or materials in H5N1 influenza A associated households and their surroundings. The Vong et al. study is support for the use of RT-PCR for H5N1 influenza A virus detection for environmental surveillance.

Poddar, Espina, and Schnurr (2002) investigated a single-step multiplex RT-PCR methodology against standard laboratory methods for influenza virus detection, type, and subtype identification. The purpose of this investigation was to determine if a single-step multiplex RT-PCR methodology could effectively reduce the time and labor required to determine influenza A and B virus types in comparison to standard culture and/or monclonal antibody-based immunofluorescence methods (Poddar et al., 2002). The authors of the Poddar et al. study asserted the single-step multiplex RT-PCR methodology is potentially applicable to succesful type and subtype detection of clinical specimens suspect for influenza virus of type A, with subtype H1N1 or H3N1, or type B virus. The Poddar et al. study is support for the use of RT-PCR for influenza A virus detection in my dissertation study.

Lu (2006) conducted a comparison of a novel dot-enzyme-linked immunosorbent assay against the commercial Directigen kit (Becton Dickson Microbiology Systems, Cockeysville, MD) to detect influenza A virus. The purpose of this study was to determine if dot-enzyme-linked immunosorbent assay showed higher sensitivity and specificity against the commercial Directigen kit (Lu, 2006). Lu asserted the sensitivity

and specificity of the novel dot-enzyme-linked immunosorbent assay was comparable against the commercial Directigen kit for the detection of influenza A virus. The Lu study is support for the use of commercially available standard of practice influenza A virus detection assays for this dissertation research.

Harmon, Bower, Kim, Pentella, and Yoon (2010) investigated a novel multiplex real-time RT-PCR to detect the matrix gene (M gene) sequence of the U.S. human pandemic H1N1 influenza A virus. The objective of this study was to develop a real-time RT-PCR test for the detection and differentiation of the 2009 pandemic H1N1 and endemic influenza A viruses in North America (Harmon, Bower, Kim, Pentella, & Yoon, 2010). The authors of the Harmon et al. study cited the M gene is known to be genetically conserved among all influenza A viruses, therefore it was selected as the target for a real-time RT-PCR assay capable of detecting all influenza A viruses and differentiating pandemic H1N1 from endemic influenza A virus directly from clinical specimens of various species origin. Further, the authors of this study asserted this newly developed real-time RT-PCR assay is rapid and the results were confirmed by other PCR tests (Harmon et al., 2010). The Harmon et al. study is support for the use of RT-PCR to target the M gene sequence for influenza A virus detection in this dissertation study.

Lee, Loh, Lee, Tang, Chiu, and Koay (2012) conducted a validation investigation of a sensitive and specific high-throughput duplex universal influenza A and B real time RT-PCR assay. The researchers of this study utilized primers and probes targeted to the matrix protein (M gene) of influenza A and the nucleoprotein of influenza B (Lee, Loh, Lee, Tang, Chiu, & Koay, 2012). The authors of the Lee et al. study stated that for

influenza A virus matrix proteins; a forward primer, a reverse primer, and a direct probe were utilized, and for influenza B nucleoproteins; a forward primer, a reverse primer, a commercial probe, and a modified probe specific for novel influenza B varient (B/Singapore/1/2011) were utilized for the assays. Further, the authors of this study concluded from the results that this universal influenza A/B assay was able to achieve better specificity and sensitivity compared to existing monoplex assays (Lee et al., 2012). The Lee et al. study is support for the use of RT-PCR to target the M gene sequence for influenza A virus detection in this dissertation study.

Magnard, Valette, Aymard, and Lina (1999) investigated two nested RT-PCR assays using previously frozen prepared nasal swap samples from outpatients presenting with acute respiratory infection during the peak influenza season of 1998. The goal of this investigation was to highlight the need for a global approach to influenza detection/culture that would encompass both WHO requirements for influenza surveillance and laboratory management for rapid diagnosis of influenza (Magnard, Valette, Aymard, & Lina, 1999). The PCR-1 assay for this investigation was a multiplex RT-PCR designed to detect specifically the haemagglutinin genes of influenza A H1N1, A H3N2, and influenza B viruses the PCR-2 assay was used to detect the M gene of influenza A viruses only (Magnard et al., 1999). The authors of the Magnard et al. study asserted the findings of the research successfully demonstrated the nested RT-PCR assays of this study is more sensitive screening method than IC-ELISA, and comparable to an optimised culture methodology. Further, the authors noted the nested RT-PCR assays of this study can yield results in 24 hours, whereas the optimised culture methodology

requires up to four days (Magnard et al., 1999). The Magnard et al. study is support for the use of RT-PCR to target the M gene sequence for influenza A virus detection and the use of frozen samples in this dissertation study.

Daum et al. (2007) investigated a single-step/reaction vessel format real-time RT-PCR for the detection of influenza A virus subtypes H1, H3, and H5; and influenza B virus. The goal of this study was to develop the single-step/reaction vessel format real-time RT-PCR, and evaluate the detection of influenza A virus subtypes H1, H3, and H5; and influenza B virus against well established conventional "gold standard" procedures for viral identification in the clinical setting (Daum et al., 2007). The researchers of the Daum et al. study used primer/probes to target the highly conserved region of the matrix protein (M gene) for all 16 influenza A virus subtypes and two influenza B lineages (B/Victoria and B/Yamagata). The authors of this study asserted the study results demonstrate high specificity and sensitivity in the absence of cross-reactivity (Daum et al., 2007). The Daum et al. study is support for the use of single-step real-time RT-PCR to target the M gene sequence for influenza A virus detection in this dissertation study.

Heijnen and Medema (2011) investigated ultrafiltration methods along with real-time quantitative reverse transcriptase PCR (qRT-PCR) assays to analyze samples of sewage influent, effluent, and surface water for the pandemic influenza A (H1N1) 2009 virus. The samples were collected in the Netherlands during the time of the influenza A (H1N1) 2009 virus epidemic (Heijnen & Medema, 2011). The researchers of the Heijnen and Medema study used water concentrates that were artifically contaminated with the pandemic influenza A (H1N1) 2009 virus as postive controls for the qRT-PCR

methodologies. The researchers of the Heijnen and Medema study used primers and probes specifically targeted for all influenza A viruses and specifically for the pandemic influenza A (H1N1) virus. The authors asserted the results of this study confirm the suitability of the laboratory methods to concentrate influenza virus from sewage and surface water and demonstrates the applicability of the qRT-PCR method to detect influenza virus in environmental samples (Heijnen & Medema, 2011). The Heijnen and Medema study is support for the analysis of environmental water samples for influenza A viruses using PCR methodologies in this dissertation study.

Agüero, San Miguel, Sánchez, Gómez-Tejedor, and Jiménez-Clavero (2007) investigated fully automated high-throughput real-time RT-PCR methodology against conventional manual real-time RT-PCR methodology for the detection of influenza A viruses. The goal of this study was to provide a rapid methodology for influenza A virus genome and the differentiation of H5N1 variants in a high number of samples (Agüero, San Miguel, Sánchez, Gómez-Tejedor, & Jiménez-Clavero, 2007). The researchers of the Agüero et al. studied field samples of cloacal/fecal and tracheal/oropharyngeal swabs, tissue homogenates, and feces from birds. In this study, for the detection of avian influenza virus the researchers targeted the matrix protein (M gene) (Agüero et al., 2007). The authors of the Agüero et al. asserted the results of the automated high-throughput real-time RT-PCR methodology of this study provides higher sensitivity and specificity when compared to traditional nucleic acid methods. Further, the authors propose the automated high-throughput real-time RT-PCR methodology allows for the analysis of large numbers of samples with fast turnaround time making it feasible for the screening

of virus circulation in animal populations at large scale (Agüero et al., 2007). The Agüero et al. study is support for the use of RT-PCR to target the M gene sequence for influenza A virus detection in this dissertation study.

Infectivity

Brown et al. (2009) investigated the infectivity of 12 low pathogenic avian influenza viruses across various pH, salinities, and temperatures in a biosafety level 2 laboratory setting. The researchers of this study utilized MDCK cell line to determine endpoint titers for infectivity of the different viruses (Brown et al., 2009). The authors of the Brown et al. study reported that endpoint titers for infectivity were measured at 100% monolayer destruction of the MDCK cells.

In the study by Brown, Swayne, Cooper, Burns, and Stallknecht (2007), a similar laboratory method using MDCK cell line was used for low pathogenic avian influenza A virus and high pathogenic avian influenza A virus infectivity assays. In this this study, endpoint titers were measured at 100% monolayer destruction of the MDCK cell line (Brown, Swayne, Cooper, Burns, & Stallknecht, 2007). Faust et al. (2009) investigated the biological effect of filter-feeding bivalves to remove influenza viruses from water and to reduce viral infectivity. The researchers of this study reported that endpoint titers for infectivity was measured at 100% monolayer destruction of the MDCK cells. These studies are support for the use of MDCK cell line protocols for infectivity assay for this dissertation research.

Gene Sequencing

Gene sequencing *or nucleotide sequencing*, and phylogenetic analysis of isolated influenza A viruses has been a source of data from where researchers have hypothesized the origins and sources of the components of the viral RNA sequences. Yamamoto et al. (2011) investigated fecal samples from migratory waterfowl from Siberia for the presence of H5 HA and N1 NA genes of influenza viruses. Nulceotide sequencing was performed on samples positive for H5 HA and N1 NA genes (Yamamoto et al., 2011). The authors of the Yamamoto et al. study concluded the results of nucleotide sequencing indicate highly pathogenic avian influenza H5N1 virus had not become dominant in the nesting lakes of Siberia prior to this study. The Yamamoto et al. study is support for the importance and usefulness of genetic sequencing of isolated H5 HA and N1 NA viruses in this dissertation study. Further, the Yamamoto et al. study associated viral presence in nesting lakes in Siberian to urban ponds in Hokkaido, Japan via migratory flyways. This migratory flyway association were of importance to mapping of the burden of influenza A viruses in rural and urban ponds in this dissertation.

Zhang et al. (2011) collected 200-ml water samples from areas near the habitat of migratory birds in East Dongting Lake, Yueyang City, Hunan Province for laboratory analysis. The authors of the Zhang et al. study asserted the Dongting Lake wetland as an important habitat and over-wintering area for East Asian migratory birds. From the 95 water samples collected, an H10N8 influenza A virus was isolated, whole genome genetic sequenced, and pathogenicity investigated in mice and specific pathogen free (SPF) White Leghorn Chickens (Zhang et al., 2011).

The phylogenic analysis of the isolated H10N8 virus indicated all the eight gene segments were of aquatic avian origin and belonged to a Eurasian lineage (Zhang et al., 2011). The authors of the Zhang et al. (2011) study concluded the H10N8 subtypes of avian viruses might pose a potential threat to mammals and multiple amino acid substitutions are liklely to be involved in the adaptation of H10N8 influenza virus to mice. This study supports environmental water sampling approach for influenza risk assessment of this dissertation research. Notably, the researchers of the Zhang et al. collected 200-ml water samples in areas near the habitat of migratory birds and stored the samples at -80°C until assayed. Similar sampling methods were used in this dissertation study.

Fouchier, et al. (2005) isolated influenza A viruses from black-headed gill (Larus ridibundus) in Sweden. These isolated influenza A viruses could not be classified to the 15 known HA subtypes. The authors hypothesized the HA of the black-headed gull viruses represent a new HA subtype (Fouchier, et al., 2005). The researchers of the Fouchier, et al. study applied nucleotide sequence analysis, phylogenic analysis, and double immunodiffusion assay methods to the ten samples from black-headed gulls. The results of the assay methods were further analyzed with BLAST available from GenBank (Fouchier, et al., 2005). The authors of the Fouchier, et al. study asserted the results provide strong evidence for the classification of the HA of the Swedish black-headed gull virus as H16. The Fouchier et al. study is support for nucleotide sequence analysis and BLAST analysis from GenBank in this dissertation study.

Tang, et al. (2009) investigated the biological properties and virulence of four H5N1 influenza subtypes [A/mallard/Huadong/Y/2003 (Y), A/mallard/Huadong/hn/2005 (hn), A/mallard/Huadong/S/2005 (S), and A/mallard/Huadong/lk/2005 (lk)] were investigated in different animal models. The results of the animal models showed all four H5N1 influenza strains to be highly pathogenic in specific pathogen free (SPF) chickens; the (S) and (Y) isolates were moderately pathogenic in mice; and in mallard ducks, two isolates (Y & hn) had low pathogenicity, and the other two (S & lk) had high pathogenicity and caused lethal infection (Tang, et al., 2009).

The researchers of the Tang, et al. (2009) study further sequenced the whole genome of the four H5N1 influenza subtypes and compared the findings to other H5N1 sequences available in GenBank. The results of this study supports the theory that influenza viruses that were previously transmissible in one species can infect a broader range of hosts by reassortment (Tang, et al., 2009). The authors of the Tang, et al. study stressed more and more H5N1 viruses are capable of causing disease and mortality in ducks, and there is a need to understand the molecular basis of the two viruses with different pathogenicity for ducks. The Tang, et al. study is support for the investigation of H5N1 virus subtypes in aquatic environments in this dissertation research.

Tønnessen, et al. (2013) studied influenza viral swabs collected from hunter-harvested dabbling ducks and gull in the south-west of Norway for the period of 2005 to 2010. Norway has the longest coastline in Europe and is located along the East Atlantic flyway of migratory waterfowl (Tønnessen, et al., 2013). The authors of the Tønnessen, et al. study conveyed the purpose of this research to: (i) describe the prevalence and

subtype diversity of avian influenza viruses in dabbling ducks and gulls in the south-west (SW) of Norway, (ii) test whether the virus prevalence was influenced by host species and sampling time, (iii) determine the genetic similarity of the Norwegian avian influenza viruses to previously characterized avian influenza viruses, and to (iv) search for evidence of intracontinental and intercontinental reassortment of gene segments encoding the internal viral proteins in avian influenza viruses from gulls in Eurasia.

Influenza RNA extraction, virus isolation, hemagglutination assay, genomic sequencing, similarity searches, and phylogenic analysis were performed on the samples (Tønnessen, et al., 2013). The authors of the Tønnessen, et al. (2013) study presented collected data in tables and figures. The phylogenic relationships of the internal gene segments are presented in a phylogenic tree diagrams. The phylogenic tree diagrams provide a visual supplement to the discussions of intracontinental reassortment and intercontinental reassortment. The authors of the Tønnessen, et al. asserted the results indicate that gulls that interact with dabbling ducks are likely to be mixing vessels for avian influenza viruses from waterfowl and gulls. Additionally, the results indicated intercontinental reassortment is rare in avian influenza viruses from gulls in Eurasia (Tønnessen, et al., 2013). The Tønnessen, et al. study is support for mixing vessel theory, sampling along migratory flyways for influenza viruses, genome sequencing, and similarity analysis of the findings in this dissertation study.

Borm et al. (2011) conducted an in-depth analysis of the complete genome sequences of three avian influenza viruses co-occurring in wild mallards at a single location at the same time was conducted. The influenza samples were collected from

trapped mallards as part of a long-term wild bird monitoring program on a pond in La Hulpe, a nature reserve approximately 20 km southeast of Brussels, Belgium (Borm et al., 2011). The authors of the Borm et al. study stated La Hulpe is visited during the migration period by mallards originating from the north and northeast (Fenoskandia and Western Russia), using the site as a stopover to more southern wintering areas. The characterization of three avian influenza viruses co-occurring in wild mallards at a single location at the same time provides a baseline measure for the long-term wild bird monitoring program. The Borm et al. (2011) study is support for genome sequencing as part of baseline measurements for aquatic environments visited by migratory waterfowl in this dissertation study.

Mishra, et al. (2009) investigated the genetic characterization of an H5N1 viral isolate from Manipur, India from 2007. The purpose of this investigation by Mishra, et al. was to understand the relationship of the H5N1 viral isolate to other H5N1 isolates and to trace the possible source of introduction into the country. Hemagglutination and Hemaggultination inhibition assays were performed for identification of the Manipur isolate (Mishra, et al., 2009). The researchers of the Mishra, et al. used WHO recommended diagnostic primers sets specific for influenza A HA (H5) and NA (N1) genes. Additionally, the researchers conducted whole genome sequencing and phylogenic analysis of H5N1 viruses (Mishra, et al., 2009).

The authors of the Mishra, et al. (2009) study reported the phylogenic analysis of 41 whole genomes of all eight gene segments concatenated showed the Manipur isolate was unique in the clade 2.2. All influenza A viruses that caused outbreaks in China,

Europe, Middle-East, and Africa regions are grouped in genotype Z, clade 2.2 (Mishra, et al., 2009). The authors of the Mishra, et al. study asserted the phylogenetic characterization of the Manipur isolate is evidence the H5N1 virus isolated in 2007 in India was a unique varient and not related to the 2006 Indian isolate. The Mishra, et al. study is support for the application of genome sequencing and phylogenic analysis as part of national and international surveillance programs.

Shan et al. (2010) studied animal models in part to investigate the biological and genetic characteristics of influenza A/Eurasian coot/WA/2727/79 (H6N2) virus. For genetic characterization, the researchers used primers for amplification of the HA, NP, and NA genes for A/Eurasian coot/WA/2727/79 (H6N2) virus (Shan et al., 2010). The authors reported that similarity analysis to GenBank showed the HA sequence obtained in this study to be identical to the HA sequence for this virus submitted previously (accession number CY028243) (Shan et al., 2010). Additionally, the authors of the Shan et al. manuscript asserted that this study documented the biological and genetic characteristics of an H6N2 subtype virus of wild-bird origin which are consistent with those of other LPAI viruses. The Shan et al. study is support for the investigation and analysis of influenza A viruses among waterfowl for it may provide useful information on the spectrum of avian influenza viruses existing in wild bird populations around the world.

In the Soda, et al. (2008) study, researchers investigated the applicability of a virus strain library for vaccine preparation. For the model, an H5N1 virus strain [R/(Dk/Mong-Dk/Mong)] was selected for whole virus vaccine preparation (Soda, et al.,

2008). The researchers of the Soda, et al. study isolated 524 influenza strains from 10,549 fecal samples of migratory birds. The 10,549 fecal samples were collected from waterfowl in 1996-2007 in Siberia, Mongolia, China, Australia, and Japan (Soda, et al., 2008). From the 524 influenza strains isolated in the Soda, et al. study, 14 were identified H5 influenza viruses. The researchers evaluated the relationships among the H5 influenza virus strains and the nucleotide sequences of the HA genes were determined and compared to those of H5 viruses from the NCBI database. To assess the potency of the R/(Dk/Mong-Dk/Mong) vaccine, mice models (case-control) were H5 HPAI challenged (Soda, et al., 2008). The authors of the Soda, et al. study reported that all of the control mice died within nine days after challenged, and survival rate was correlated to vaccine dose. The Soda, et al. study is support for the establishment of a library of vaccine strain candidates from isolates of waterfowl. The establishment of a library database may be of biological importance to vaccine development and influenza risk assessment tools.

Influenza Risk Assessment Tools

The ultimate goal for epidemiologists and other influenza researchers is to identify pandemic influenza viruses before they emerge in animal or human populations so that interventional strategies may be implemented that would prevent or reduce the impact of the influenza outbreak (Keeler, 2011). Doherty and McLean (2011) included several conceptual and practical issues associated to monitoring influenza virus in wild animal populations. This work includes discussions of the philosphical approaches to monitoring, design and methodological issues, probabilities of detection, and a review of several current monitoring plans (Doherty & McLean, 2011). Several of the monitoring

plans share similar characteristics to the Centers for Disease Control and Prevention Influenza Risk Assessment Tools.

CDC and ECDC IRAT

The CDC IRAT being developed consisted of ten criteria grouped within three overarching categories: (i) properties of the virus, (ii) attributes of the population, and (iii) ecology and epidemiology (Centers for Disease Control and Prevention , 2012). The ten evaluation criteria are: (i) Genomic variation, (ii) Receptor binding, (iii) Transmission in laboratory animals, (iv) Antiviral treatment susceptibility/resistance, (v) Existing population immunity, (vi) Disease severity and pathogenesis, (vii) Antigenic relationship to vaccine candidates, (viii) Global distribution in animals, (ix) Infection in animal species, and (x) Human infections. The European Centre for Disease Prevention and Control (ECDC) was developing an IRAT similarly to the CDC model.

The ECDC IRAT model is heavily focused on the human features of influenza outbreaks. The ECDC IRAT model has three overarching categories: (i) Basic epidemiology and basic parameters of human infection, (ii) Disease characteristic in humans, and (iii) Features of the virus in infected persons (European Centre for Disease Prevention and Control, 2009). At the time of this writing, the ECDC IRAT model primarily focused on attributes of the population, and does not emphasize properties of the virus or ecology and epidemiology of the virus.

New World Organization for Animal Health Standards of Reporting

Thiermann (2007) presented a summary of new international standard on avian influenza as developed by the World Organisation for Animal Health (OIE). This work

provides a summary of international efforts to control and monitor for High and Low Pathogenic Avian Influenza viruses. The importance of the work by World Organisation for Animal Health is the international uniformity in reporting standards and the support provided to poor countries where avian influenza is endemic.

Geographical Ranking for Highly Pathogenic Avian Influenza

Gonzales et al. (2010) investigated and wrote a critical review of the active surveillance of avian influenza among European Union countries. These critical reviews identified methodologies used by the international community to monitor the presence of influenza among poultry and waterfowl. These studies provide validation to research surveillance methods used to study of influenza virus infected birds and also provide support for further research to understand influenza at the human-animal interface.

Ranking of Vectors for Highly Pathogenic Avian Influenza

Carver (2003) crafted a risk assessment strategy for the transmission of highly pathogenic avian influenza (HPAI) among commercial poultry flocks. The importance of this work is the recognition and ranking of transportation vectors for risk. From calculating risk assessment to transportation vectors, biosecurity measures may be implemented to reduce risk and still maintain national and international commerce.

Mathematical Model for Pre- and Postinfection of Bird Flocks

Hollingsworth (2009) investigated previous influenza outbreaks by applying mathematical modeling. This article provides mathematical modeling technique and detailed definition of terms so that epidemiologists and other influenza researchers can further apply the principles. Hollingsworth concluded that mathematical modeling of

influenza outbreaks is an essential tool for understanding and controlling directly transmissible pathogens.

Social Change Implications

The social change implications of this dissertation can be recognized at the national and international levels, to the population level, and to the individual level. At the national and international level I attempted to contribute geospatial analysis and spatial-temporal data for influenza A surveillance. H5N1 influenza virus surveillance may detect highly pathogenic avian influenza virus H5N1 presence along the eastern Pacific flyway. The future detection of highly pathogenic avian influenza virus H5N1 may initiate biosecurity measures to protect poultry industries in the United States and Brazil. The United States and Brazil are the world's largest exporters of poultry (Butler & Ruttimann, 2006). Additionally, I attempted to contribute new H5N1 viral genome sequences to the current influenza A virus H5N1 database. An increased database may be a resource for avian influenza A virus H5N1 vaccine development.

Vaccine developments were positive social change at the population level. In the Soda, et al. (2008) study, researchers investigated the applicability of a virus strain library for vaccine preparation. I attempted to contribute to the current virus strain library at GenBank. Vaccine development may have may positive social change for American Indian/Alaskan Natives. The Centers for Disease Control and Prevention (2009) reported American Indian/Alaskan Natives had H1N1 mortality rates four times higher than persons in all other racial/ethnic populations combined. Poverty and poor living conditions may predispose American Indian/Alaskan Natives to influenza complications

(Centers for Disease Control and Prevention, 2009). Chakraborty et al. (2008) suggested the a disproportionate share of a developing country disease burden is due to infectious diseases. Applying this hypothesis to American Indian/Alaskan Natives, whose H1N1 mortality rates were four times higher than persons in all other racial/ethnic populations combined, may explain the poverty and poor living conditions that may predispose this population to influenza complications. At the individual level the results of this dissertation may bring about greater awareness of influenza A virus infection.

Shive and Kanekar (2011) suggested the principles of the Health Belief Model can provide explanation why many people, including high risk individuals, do not receive influenza vaccination. The results of this dissertation study may bring greater awareness to the individual, and thus, greater perceived susceptibility to influenza A virus infection. In this dissertation study, I analyzed samples from artificial recirculating water ponds.

Artificial recirculating water ponds are often a decorative feature in parks and golf courses. The decorative feature is often constructed to attract people. These decorative features may also attract both domestic and migratory waterfowl (Webster et al., 1992). Migratory waterfowl likely will introduce influenza A viruses to the artificial recirculating water pond enivronment from viral shedding in feces.

If the artificial recirculating water pond is constructed with an aeration fountain, mist and droplet formation may occur. As is well established, influenza viruses are transmitted between individuals through droplets (e.g., coughing, sneezing). An individual who comes in contact with the droplets from the contaminated artificial recirculating water pond may be inoculated with influenza A virus. Therefore, the

findings of this dissertation study may bring greater awareness at the individual level, and thus, increase the perceived susceptibility to influenza A virus infection. As implied by Shive and Kanekar (2011), perceived susceptibility to influenza A virus infection will have a direct correlation to influenza vaccination.

Major Themes in the Literature

The major themes in the literature are aligned to the epidemiological triangle for influenza viruses. The major theme of the *environment* is continued surveillance. The Centers for Disease Control and Prevention, European Centre for Disease Prevention and Control, Chinese Center for Disease Control, and the World Health Organization are in agreement that continued influenza surveillance will lead to reduced morbidity and mortality from influenza virus infection. The literature review revealed many studies support and contribute to influenza surveillance. A portion of this literature has focused on the role of migratory waterfowl.

The major theme of the *host* is migratory waterfowl are the natural reservoir of influenza viruses and the cause of global dispersal of influenza viruses. Investigations by others have concentrated and pinpointed to migratory waterfowl breeding grounds, global migratory flyways, and the phylogenic analysis of influenza viruses isolated from migratory waterfowl. Phylogenic analysis investigations used molecular techniques and the GenBank data base to flesh out the evolutionary patterns and changes of the influenza viron. These discoveries support Mixing Vessel Theory. Another application of molecular techniques was to characterize isolated influenza viruses.

The major theme of the *agent* is the influenza viron is not completely understood. Studies have attempted to discover characterizations of influenza virus strains that are pathogenic in some animals and non-pathogenic in others. Other studies have attempted to better understand the molecular level dynamics of the influenza viron that leads to animal-to-human transmission (primary) and human-to-human transmission (secondary). Another field of studies has investigated vaccine development against the mutable influenza virus. The major themes of the literature shape an organization to what is known and what is not known about the global influenza burden.

What Is Known as Well as What Is Not Known

There are subjects with in the topic of the influenza that are known and accepted in the literature. First, in the literature migratory waterfowl and shorebirds are recognized as natural reservoirs for influenza viruses. Second, antigenic drift is accepted as a causative process from which influenza viruses can evade humoral immunity. Third, antigenic shift is accepted as a causative process which may lead to pandemic influenza viral strains. Fourth, the Mixing Vessel Theory is widely accepted as the process of creating novel influenza strains that are capable of host switching. Fifth, the molecular structure and the RNA genome of the influenza viron are well documented. Sixth, the influenza virus genome database (GenBank) is available as a resource library to the international community and also a repository where new contributions can be made. However, there are subjects within the topic of the influenza that are not well understood in the literature.

The process of antigenic shift and antigenic drift is not well understood in the literature. This process of creating novel influenza strains and sub-type(s) of existing strains may result in viruses with molecular characteristics that allow for animal-to-human transmission, or human-to-human transmission. The molecular characteristics that give an influenza virus the properties for animal-to-human transmission, or more seriously, human-to-human transmission are not completely understood. In addition to having the properties of primary or secondary transmission, mutated influenza viruses may express varying virulence in different species.

The determinants of influenza virus virulence are not completely understood. The determinants of influenza virus virulence among migratory waterfowl need further investigation. An infected asymptomatic migratory waterfowl may host and disperse a highly pathogenic influenza strain along the migratory flyways. There is a lack of understanding of the association of which species of waterfowl can host which influenza viral strains and cause viral shedding along which migratory flyways and watershed breeding grounds. To investigate this conundrum, geospatial analysis and spatial-temporal distribution studies may be of significant interest. In this dissertation, I attempted to fill the gap in the knowledge of the geographical dispersal of influenza A viruses in aquatic habitats using geospatial analysis.

Chapter 3: Research Method

The "Spanish" influenza H1N1 pandemic of 1918–1919, which caused approximately 50 million deaths worldwide, remains an ominous warning to public health (Taubenberger & Morens, 2006). Since then, new subtypes of human influenza A viruses have been detected at various times: in 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, the H3N2 subtype (Hong Kong virus) appeared; and in 1977, the H1N1 virus reappeared (Webster, et al., 1992). Influenza disease emergence data are collected year-round, but economic strain on global public health to prevent and treat human influenza outbreaks is enormous. Therefore, it is imperative to identify potential sources of the virus to help minimize outbreak occurrence.

The problem in this study was that public health scientists have been battling emerging human influenza diseases with tactile and reactionary methods because there was a lack of knowledge and data at the human-animal interface. This baseline study of the proportion of influenza A virus in urban and rural community settings may provide knowledge and biological data of significant interest at the human-animal interface. This biological data may be of significant interest for the development of IRAT.

Organization of the Chapter

This chapter includes the materials and methodology of this dissertation study. The underpinning organization of materials and methodology of this dissertation was the epidemiological triangle as it pertains to influenza A viruses in the aquatic habitat. The organization of this chapter begins with the research design and rationale. This section is followed by presentation of the research questions and the connection to the research

design. Succeeding this section is a presentation of the research design connection to the literature. The methodology of the sample selection, pre-analytical, analytical, and post-analytical phases of the study follows. The research questions are again presented for the ease of the reader. This is followed by a presentation of the statistical analysis approaches to each research question and the threats to validity. The ethical procedures of the study and summary are presented to conclude the chapter.

Research Design and Rationale

For this quantitative dissertation study, I collected and analyzed primary data. The epidemiological triangle provided framework for the primary data collected. The epidemiological triangle has three vertices: host, agent, and environment. The host is the organism harboring the disease. For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*; Franklin et al., 2011). The agents are the influenza A viruses. The environments are the natural and artificial habitats where the hosts are found.

The independent environmental variables of this study were the geographical locations of the artificial water ponds and geochemical properties of the artificial water pond samples. The geographical location data included rural or urban community, latitude and longitudinal coordinates, altitude, and approximate water pond surface area. The geochemical properties of the artificial water pond sample measure included pH, salinity, and temperature. Additionally, the presence of absence of waterfowl at the time of sample collection was included as an independent host variable. The dependent agent variables were influenza A viruses. Influenza A virus data included detection for M gene by real

time RT-PCR using World Health Organization recommended primer sequences, infectivity by hemagglutination assay using MDCK cell line, and gene sequencing for H5N1 influenza A virus using World Health Organization recommended primer sequences at Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California).

Researchers have recognized the human-animal interface as a complex but critical juncture at which new paradigms have been emerging (World Health Organization, 2011). The aquatic virus reservoir is a human-animal interface. Franklin et al. (2011) suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals. The quantitative research design of this dissertation was used to investigate aquatic virus reservoirs as a human-animal interface. Further, the quantitative design of this dissertation sought to answer the research questions.

Research Questions

R1: Is there a difference in the burden of influenza A virus in rural ponds compared to urban ponds?

$H_0$1: The burden of influenza A virus in community ponds has no association to geographical location.

H_a1: The burden of influenza A virus in community ponds is associated to geographical location.

In this study the proportion and probability of the presence of influenza A virus were investigated in recirculating artificial ponds in rural and urban geographical

locations. Rural ponds were viewed as one population and urban ponds as another population. The dependent agent variables were influenza A viruses.

R2: Is there an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds?

$H_0$2: There is no association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

H_a2: There is an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

In this study, rural community ponds were viewed as one population and urban community ponds as another population. The comparison of these two populations included proportion of influenza A virus(s), latitude and longitudinal coordinates, altitude, and approximate water pond surface area, and the presence of absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) at the time of sample collection were included as an independent environment and host variables. The dependent agent variables were influenza A viruses.

R3: Is there an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds?

$H_0$3: There is no association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

H_a3: There is an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

Brown et al. (2009) investigated the infectivity of 12 low pathogenic avian influenza viruses across various pH, salinities, and temperatures in a biosafety level 2 laboratory setting. In this dissertation study, I investigated if the findings of the Brown et al. study were consistent in rural and urban community pond samples. The geochemical properties of the artificial water pond sample measure included: pH, salinity, and temperature. The dependent agent variables were infectivity influenza A viruses.

R4: Are H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds novel gene sequences or have the gene sequences been previously identified elsewhere?

$H_0$4: The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are not novel gene sequences and the gene sequences have been previously identified elsewhere.

H_a4: The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are novel gene sequences and the gene sequences have not been previously identified elsewhere.

In this study, water samples were collected directly from rural and urban ponds. World Health Organization recommended RT-PCR primers were used for influenza A virus H5N1 gene sequencing. The laboratory analysis for the presence of influenza A virus and influenza A virus infectivity were conducted in a biosafety level 2 laboratory at the California State University Channel Islands campus. The analysis for the presence of

influenza A virus involved commercially available influenza A/B viral RT-PCR detection kits suitable for water analysis. Water samples verified positive for H5N1 influenza A viral strains were submitted for gene sequencing analysis to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California). The H5N1 influenza A virus gene sequencing results were compared to the influenza A viral gene sequence database GenBank (National Institute of Health genetic sequence database).

Time and Resource Constraints

Applied environmental studies can be classified as baseline, monitoring, or impact studies (Green, 1979). This dissertation is a baseline environmental study. Green (1979) asserted a *baseline study* is one in which data are collected and analyzed for the purpose of defining the present state of the biological community, the environment, or both (p. 68). In this dissertation study, I attempted to define the present state of the biological community of recirculating artificial ponds in rural and urban geographical locations in California. The temperate climate of California did not pose seasonal constraints. However, to define the present state of the biological community, a cross-sectional sampling approach were used. To limit threats to the internal validity of the study, pond water samples were collected over the shortest time period, as possible. At the time of this writing, there were no resource contraints apparent.

Research Design Choice

Water analysis has been an important scientific approach to understanding the epidemiological triangle model of influenza viral diseases. Ferro et al. (2010) focused on the presence of influenza A viruses among the migratory ducks and the winter breeding

grounds along the Texas Coast. Studies by others have investigated waterborne transmission of influenza as a zoonotic disease in the natural environment (Lang et al., 2008; VanDalen et al., 2010; Webster et al., 1992). VanDalen et al. (2010) were able to show viral sheding in fecal material to be of higher viral concentrations than oropharyngeal or cloacal swabs. Thus, it is possible that influenza viruses can be transfered between organisms via open water-sources as wetlands, lakes, and ponds.

Other researchers have investigated if influenza A viruses can be detected in these contaminated aquatic habitats (Lang et al., 2008; Stallknecht & Brown, 2009; Zhang et al., 2006). Lang, et al. (2008) studied sediment sample from three ponds in the Creamer's Field Migratory Waterfowl Refuge, Alaska, a location used by a wide variety of migratory waterfowl. The sediment samples were collected using a time-series approach and analyzed for influenza A virus RNA using reverse transcription-polymerase chain reaction (RT-PCR) methodologies (Lang et al., 2008).

Zhang et al. (2006) studied samples of ice or water from three northeastern Siberian lakes in the Koluma River region collected and analyzed for the presence of influenza A virus using reverse transcription-polymerase chain reaction (RT-PCR) methodologies. Other researchers have studied influenza A viruses in surface water samples to investigate the perpetuation between waterfowl and water-contamination (Hinshaw et al., 1980; Ito et al., 1995), while others have researched the infectivity and transmission of influenza A viruses in the aquatic environment (Brown et al., 2009; Halvorson et al., 1985).

Ito et al. (1995) investigated influenza virus isolates they collected fecal samples of dabbling ducks and also from lake water used by migratory waterfowl. The purpose of this study was to investigate the theory of water-borne transmission of influenza virus (Webster et al., 1992) as a mechanism of year-to-year perpetuation of the viruses in lakes where migratory waterfowl may breed (Ito et al., 1995). The results of this research support the hypothesis the viruses remained viable in the lake water after most ducks left for migration South (Ito et al., 1995). Brown et al. (2009) asserted the pH, temperature, and salinity-at levels normally encountered in nature-can impact the ablity of avian influenza A viruses to remain infective in water.

In the longitudinal study by Hinshaw et al. (1980), researchers collected and analyzed samples from waterfowl, unconcentrated lake water, and feces from lake shores near Vermillion, Alberta, Canada to isolate influenza viruses. The samples were used to investigate whether influenza viruses continually circulate or whether the same or different strains are present from year to year (Hinshaw et al., 1980). The authors of the Hinshaw et al. (1980) study asserted this longitudinal study of feral ducks would provide data on the size of the gene pool of influenza A viruses in nature and the number of viruses antigenically related to human strains present in avian species.

Methodology

Artificial water ponds in the geographic locations of rural and urban communities are sites of the human-animal interface. Webster et al. (1992) described this human-animal interface with the example of domestic ducks in community ponds attracting migratory waterfowl. The migratory waterfowl introduce the influenza virus to that

community's water pond from fecal contamination. The contaminated community water pond now becomes a potential source of influenza virus to both humans and animals.

Population

The target population for this dissertation was artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. There are 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l). The population size of artificial recirculating water ponds in California is unknown.

Sampling and Sampling Procedures

The dissertation study method was quantitative using a cross-sectional design. A convenience sampling approach was used. The geographical area was the state boundaries of California. Equal sample sizes from rural and urban communities were attempted. A representative sampling from each of the 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l) were attempted. The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban communities.

The number of research articles that have either focused on, or have included water sample analysis for influenza viruses has been limited (Runstadler, Hill, Hussein, Puryear, & Keogh, 2013). Henaux, Samuel, Dusek, Fleskes, and Ip (2012) collected water samples (n = 597) from wetlands of Sacramento Valley, California and Yolo Bypass, California and analyzed them for the presence of avian influenza virus. The

overall detection rate for avian influenza virus in the Henaux et al. study was [12/597 = 2.0 ± 0.6%]. For water samples collected from wetlands of Sacramento Valley, California, the detection rate for avian influenza virus was [2.7%] and for water samples collected from wetlands of Yolo Bypass, California, the detection rate for avian influenza virus was [1.7%] (Henaux, Samuel, Dusek, Fleskes, & Ip, 2012). The authors of the Henaux et al. study asserted these findings show no difference between the two locations. The wetlands of Sacramento Valley, California and Yolo Bypass, California are designated urban areas in the California Business and Professions Code Section 19986(l). Similar results to the Henaux et al. study were found in the Ito et al. (1995) study.

Ito et al. (1995) collected water samples (n = 103) from wetlands in Alaska and analyzed them for the presence of avian influenza virus. For water samples collected from wetlands proximal to Anchorage, Alaska, the detection rate for avian influenza virus was [1/17 = 5.9%] and for wetlands proximal to Fairbanks, Alaska, the detection rate for avian influenza virus was [0/5 = 0.0%] (Ito et al., 1995). According to the State of Alaska, Department of Labor and Workforce Development (2010), Anchorage, Alaska is considered an Urbanized Area and Fairbanks, Alaska is considered an Urban Cluster. The remaining locations where Ito et al. collected water samples could not be determined is assumed to be rural municipalities (non-Urbanized Areas or non-Urban Clusters). For water samples collected from wetlands proximal to rural municipalities of Alaska, the detection rate for avian influenza was [11/81 = 13.6%]. Similar results to the Henaux et al. (2012) study were found in the Perez-Ramırez, et al. (2012) study.

Perez-Ramırez, et al. (2012) collected water samples (n = 98) from wetlands in predominantly urban areas of Spain and analyzed them for the presence of avian influenza virus. The detection rate for avian influenza virus in the Perez-Ramırez, et al. (2012) study was [0/98 = 0.0%]. Since the proportion of influenza A virus in rural and urban ponds is unknown, findings of the Ito et al. study, the Henaux et al. (2012) study, and the Perez-Ramırez, et al. study were used to support the sample size calculations for this dissertation study. The likely estimate for the proportion of influenza A virus in urban ponds were [13/717 = 1.8%]. The likely estimate for the proportion of influenza A virus in urban ponds were [11/81 = 13.6%].

The sample size formula for number to estimate prevalence in a survey is: $n = (Z_{\alpha/2}+Z\beta)^2 * (p_1(1-p_1)+p_2(1-p_2)) / (p_1-p_2)^2$. Since the proportion of influenza A virus in rural is likely estimated to be [1.8%, $p_1 = 0.018$] and urban ponds is likely estimated to be [13.6%, $p_2 = 0.136$], the minimum sample size for each will need to be [n = 77, Z = 1.96, precision (e) = ± 0.05]. Risk difference (RD), relative risk (RR), odds ratio (OR), and Cohen's g were reported posteriori.

RD, RR, and OR are common effect size indices reported for groups (the *d* family) compared on dichotomous outcomes (Ellis, 2010). Cohen's g [Cohen's g = p – 0.5, *where p estimates a population proportion*] can be used to define the effect size for a proportion (Furr, 2008). Effect size classes for Cohen's g are: [small = 0.05, medium = 0.15, large = 0.25] (Ellis, 2010). Logistic regression was used in this study and the regression coefficient was reported as a measure of effect size. Breaugh (2003) asserted the regression coefficient can be interpreted as a measure of the effect size. Further, the

regression coefficient reflects the expected change in the log of the odds associated with a one unit change in the independent variable (Breaugh, 2003).

The alpha value were [alpha = 0.05] and power level were [power = 0.80] for this study. The alpha value is the probability of making a false positive result (Type I errors) (Motulsky, 1995). Hence, rejecting the null hypothesis when in fact, the null hypothesis is true. [Alpha = 0.05] is selected to maintain continuity with the level of precision chosen for the minimum sample size for a population unknown proportion [precision (e) = ± 0.05]. The power value is a transformation [power = 1 – beta] of the probability of making a false negative result (Type II error) (Motulsky, 1995). Hence, accepting the null hypothesis when in fact, the null hypothesis is false. [Power = 0.80] is commonly used in the literature (Forthofer, Lee, & Hernandez, 2007).

Since the samples will likely be under public or private ownership, a signed Collection Site Contact Letter were offered and Letter of Cooperation were received before any field analysis or water collection were conducted (see Appendices A and B for templates of letters). Preliminary inquiry with local public officials to the acceptance of the Letter of Cooperation and feasibility of water sample collection for this dissertation study has been positive. The calculated total sample size (N_{Calc} = 154) for this dissertation study was exceeded. The actual collected samples size is (N = 182). Artificial recirculating water ponds with an approximate surface area less than 900 square feet were excluded.

Procedures for Recruitment, Participation, and Data Collection

The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. The geographical area was the state boundaries of California. Equal sample size from rural and urban communities were attempted (n = 77). A representative sampling from each of the 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l) were attempted.

The methodology for characterization of influenza viruses has shown similarity across the literature. Overarching methodologies include: virus detection by RT-PCR (Poddar et al., 2002), virus isolation and characterization by allantoic cavities of embryonic eggs (Fouchier, et al., 2005), infectivity studies using MDCK cell line (Brown et al., 2009), nucleotide sequencing of the RNA genome (Yamamoto, et al., 2011), and phylogenic analysis using BLAST available from GenBank (Fouchier, et al., 2005).

Water Sampling (Preanalytical Phase)

Water samples were collected as previously published in the *U.S. EPA Field Sampling Guidance Document #1225*. A dip sampler for water collection was used. A dip sampler is useful for situations where a sample is to be recovered from an outfall pipe or along a pond bank where direct access is limited. The long handle on such a device allows access from a discrete location so as not to disturb wildlife (U.S. Environmental Protection Agency, 1999). The water volume per sample was 200 ml. Other researchers have collected 200 ml volume water samples for influenza studies.

Zhang et al. (2011) collected 200-ml water samples from areas near the habitat of migratory birds in East Dongting Lake, Yueyang City, Hunan Province for laboratory analysis. The authors of the Zhang et al. study asserted the Dongting Lake wetland as an important habitat and over-wintering area for East Asian migratory birds. From the 95 water samples collected, an H10N8 influenza A virus was isolated, whole genome genetic sequenced, and pathogenicity investigated in mice and specific pathogen free (SPF) White Leghorn Chickens (Zhang et al., 2011). Others have investigated the abiotic environmental factors affecting avian influenza A virus persistence in aquatic habitats.

Brown et al. (2009) investigated the duration of infectivity for avian influenza viruses representing a diversity of HA subtypes (H1 – H12) isolated from waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) over a range of pH, salinity, and temperature conditions that are common in aquatic habitat environments utilized by these wild aquatic birds. The findings of this study indicate the pH, temperature, and salinity-at levels normally encountered in nature-can impact the ability of avian influenza A viruses to remain infective in water (Brown et al., 2009).

In this dissertation, I tested the laboratory findings of the Brown et al. (2009) study to artificial recirculating water ponds in the geographic locations of rural and urban communities. The pH, salinity, and temperature conditions of water samples were analyzed using an Oakton Multi-Parameter Tester 35 Series Model 35425-10 (Eutech Instruments, Thermo Fisher Scientific, Huntington Beach, California) according to manufacturer's instructions. Approximate water pond surface areas were calculated using a Carl Zeiss Victory 8 x 26 T* PFR (Carl Zeiss, Sports Optics Gmbh, Germany) laser

rangefinder for X-axis and Y-axis measurements, or a GARMIN® eTrex model 20 (Garmin, Ltd.) handheld GPS device using the "Calculate Area" function, or Google Earth estimated measurement function. Measurements of distance were taken along the long and short axis of the body of water. Approximate surface area was calculated using the formula for the area of an ellipse: Area = $\pi(x/2)(y/2)$ where x = distance of long axis of body of water, and y = distance of short axis of body of water, and $\pi = 3.141592654$. Sampling procedures were as follows:

1. Determine the extent of the sampling effort, the sampling methods to be employed, and which equipment and supplies are needed.
2. Obtain necessary sampling and monitoring equipment.
3. Decontaminate or preclean equipment, and ensure that it is in working order.
4. Prepare scheduling and coordinate with staff, clients, and regulatory agency, if appropriate.
5. Provide confidentiality agreement and obtain release for data and sample collection.
6. Perform a general site survey prior to site entry and evaluate for health and safety.
7. Measure and calculate approximate surface area of body of water.
8. Use handheld GPS unit and handheld altimeter unit to identify and mark all sampling locations. If required, the proposed locations may be adjusted based onsite access, property boundaries, and surface obstructions.
9. Assemble the device in accordance with the manufacturer's instructions.

10. Extend the device to the sample location and collect the sample.
11. Retrieve the sampler and transfer the sample to the appropriate sample container.
12. Approximately 200 ml of sample surface water were collected.
13. Transfer the sample(s) into suitable labeled sample containers.
14. Cap the container, put it in a Ziploc plastic bag and place it on ice in a cooler.
15. Perform pH, salinity, and water temperature tests.
16. Make observations for presence or absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*).
17. Make observations of location setting (park land, golf course, University campus, business district, or other).
18. Record all pertinent data in the site notebook and on a field data sheet.
19. Complete the chain of custody form.
20. Attach custody seals to the cooler prior to shipment, as needed.
21. Decontaminate all sampling equipment prior to the collection of additional samples.

Sample Preparation (Preanalytical Phase)

Sample integrity was maintained until received in the laboratory for processing. Sample identification was maintained throughout the laboratory testing. Filtering of samples removed particulate matter and other suspended contaminates. Evers et al. (2007) studied the commercial preservative RNAlater (Qiagen) by evaluating against the current method of cryo-freezing, and ethanol preservatives for influenza A virus samples.

From the findings of the Evers et al. study, the authors asserted the commercial preservative RNAlater held at ambient temperatures might be useful for the identification of influenza A virus in samples collected from infected waterfowl.

Materials required:

- Filter paper of 0.4 μm pore or smaller
- 250ml centrifuge tubes
- Microvials
- QIAamp® Viral RNA Mini Kit (QIAGEN® Cat#52904 or 52906)
- RNAlater RNA Stabilizer reagent (Qiagen)

Sampling procedures are as follows:

1. Samples were filtered using standard grade laboratory filter paper of 0.4 μm pore or smaller.
2. The filtered sample were poured into 250 ml centrifuge tubes and concentrated at 10,000 rpm for 20 minutes at 4°C using a Sorvall SL-1500 Super-Lite centrifuge rotor or equivalent.
3. Pour off supernatant, vortex, and transfer the remaining pellet by clean pipette to a polypropylene microvial. This was the influenza virus stock for the hemagglutination assay.
4. Add 100 μL of RNAlater RNA Stabilizer reagent (Qiagen) to 100 μL of influenza virus stock in a separate polypropylene microvial for viral RNA extraction.

5. Proceed to extract viral RNA using QIAamp® Viral RNA Mini Kit (QIAGEN® Cat#52904 or 52906) following manufacturer recommended protocol.
6. Store the extracted viral nucleic acid elute samples according to manufacturer's instructions.
7. Retain unused influenza virus stock (from Step 3) for Hemagglutination assay (infectivity assay).
8. Record all pertinent data in the laboratory notebook.

Hemagglutination Assay (Analytical Phase)

Virus isolation is a highly sensitive and useful technique for the identification of influenza viruses in the environment (Szretter, Balish, & Katz, 2006). Szretter, Balish, and Katz (2006) asserted influenza viruses are quantified either by a "unit" of hemagglutination, which is not a measure of an absolute amount of virus but is an operational unit dependent on the method used for the hemagglutination assay titration or plaque assay. Hemagglutination is the ability to bind red blood cells (Szretter et al., 2006). Szretter et al. asserted hemagglutination is a property of all influenza viruses that can be utilized as a rapid assay for determining the presence of virus in samples. MDCK cells (ATCC# CCL-34) are the preferred host for the isolation and characterization of influenza A and B viruses, but not influenza C viruses due to the incompatibility of sialic acid moieties on the cell surface with the viral receptor specificity (Szretter et al., 2006).

Other researchers have used hemagglutination MDCK plaque assay to investigate influenza virus infectivity. Brown et al. (2009) studied MDCK cell line to determine

endpoint titers for infectivity of twelve low pathogenic avian influenza viruses across various pH, salinities, and temperatures. Faust et al. (2009) investigated the biological effect of filter-feeding bivalves to remove influenza viruses from water and infectivity was investigated. The researchers of these studies reported endpoint titers for infectivity measured at 100% monolayer destruction of the MDCK cells (Brown et al., 2009; Faust et al., 2009). In this dissertation study, I utilized the hemagglutination properties of influenza A viruses on MDCK cells to characterize infectivity in concentrated water samples.

Materials:

- Phosphate-buffered saline (PBS) containing potassium
- Influenza virus stock (concentrated pond water sample)
- MDCK cells
- 96-well U-bottom microtiter plate (Nunc)

Procedure:

1. Pipet 50 µl PBS into wells 2 through 12 across a 96-well U-bottom plate
2. Pipet 100 µl influenza virus stock into the first column of the 96-well plate.
3. Perform a two-fold dilution series across the 96-well plate by transferring 50 µl between wells, disposing of the final 50 µl from the last well.
4. Add 50 µl MDCK cells to all wells. Tap the plate gently to mix.
5. Approximately 5 ml MDCK cells were needed per 96-well plate used in this assay.

6. Incubate 96-well plate for 60 min at room temperature (24◦ to 27◦C). *Avian red blood cells in V-well microtiter plates require 30-min incubation at room temperature, whereas mammalian red blood cells require a U-well microtiter plate with a 60-min incubation at room temperature.*
7. Observe endpoint of agglutination and record titer per 50 µl of sample.
8. Red blood cells will settle to the bottom of the U-bottom well in negative samples, while red blood cells will agglutinate in positive samples. The endpoint should be read as the last well showing complete agglutination.
9. Dispose of materials in an appropriate biological waste container.
10. Record all pertinent data in the laboratory notebook.

Real Time RT-PCR (Analytical Phase)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a powerful technique for the identification of influenza virus genomes (World Health Organization, 2007). A constellation of studies have targeted the M gene sequence of the influenza A viral genome by RT-PCR as an indicator of influenza A virus positivity (Harmon et al. 2010; Lee et al., 2012; Magnard et al.,1999; Perez-Ramırez, et al., 2012). In the analytical phase of this dissertation study, I used real time RT-PCR. Using real time RT-PCR allows for the detection of products as amplification is ongoing, allowing quantification (World Health Organization, 2007). The analytical phase of this dissertation followed World Health Organization Real-time RT-PCR Protocol 2 for influenza A virus (H5N1) detection.

Materials required:

- QIAGEN® QuantiTect®, Probe RT-PCR kit (#204443):
- 2 x QuantiTect®, Probe RT-PCR Master Mix
- QuantiTect®, RT Mix
- RNase free Water
- Primers
- Probe
- Equipment: Chromo-4 ™ Real-time PCR Detection system (BioRad)

Primers and probes:

Real-time PCR is performed by One-step RT-PCR using TaqMan® probe

Gene	Type A (M)
Clade	1, 2, 3
Primers sequences	MP-39-67For CCMAGGTCGAAACGTAYGTTCTCTCTATC (10 µM)
	MP-183-153Rev TGACAGRATYGGTCTTGTCTTTAGCCAYTCCA (10 µM)
Probe sequences	MP-96-75ProbeAs FAM-ATYTCGGCTTTGAGGGGGCCTG-MGB (5pmol/µl)

Gene	H5
Clade	1, 2, 3
Primers sequences	H5HA-205-227v2-For CGATCTAGAYGGGGTGAARCCTC (10 µM)
	H5HA-326-302v2-Rev CCTTCTCCACTATGTANGACCATTC (10 µM)
Probe sequences	H5-Probe-239-RVa FAM-AGCCAYCCAGCTACRCTACA-MGB (5pmol/µl)
	H5-Probe-239-RVb FAM-AGCCATCCCGCAACACTACA-MGB (5pmol/µl)

Gene	N1
Clade	1, 2, 3
Primers sequences	N1-For-474-502-v2 TAYAACTCAAGGTTTGAGTCTGTYGCTTG (10 µM)
	N1-Rev-603-631-v2 ATGTTRTTCCTCCAACTCTTGATRGTGTC (10 µM)
Probe sequences	N1-Probe-501-525-v3 FAM-TCAGCRAGTGCYTGCCATGATGGCAMGB (5pmol/µl)

Real time RT-PCR procedures are as follows:

Extract viral RNA from clinical specimen with QIAamp® Viral RNA Mini Kit or equivalent extraction kit according to manufacturer's instructions.

Reaction Mixture:

- 2x QuantiTect® Probe RT-PCR Master Mix 12.5 µl
- Forward Primer (10µM) 1.5 µl
- Reverse Primer (10µM) 1.5 µl
- *Probe (5pmol/µl) 0.5 µl
- QuantiTect® RT Mix 0.25 µl
- RNase free Water 3.75 µl
- Total 20 µl

**For the reaction of H5 detection, a mixture of two probes is used.*

- H5-Probe-239-RVa 0.375 µl
- H5-Probe-239-RVb 0.125 µl

Procedure:

1. Dispense 20 µl of the reaction mixture to each RT-PCR reaction plate.
2. Add 5 µl sample RNA to the reaction mixture. For control reactions, use 5 µl of distilled water for negative control, and 5 µl of appropriate viral RNAs for positive control.
3. Program the thermal cycler according to the program outlined in below.
4. Start the Real-time RT-PCR program while RT-PCR reaction plates are still on ice.
5. Wait until the thermal cycler has reached at 50 °C.
6. Then place RT-PCR reaction plates in the thermal cycler.
7. Record all pertinent data in the laboratory notebook.

RT-PCR temperature-cycling conditions:

1. Reverse transcription 50°C 30min.
2. Denaturation 95°C 15min.
3. PCR 94°C 15sec.
4. 56°C 1min. [45 cycles]

Record all pertinent data in the laboratory notebook.

Genetic Sequencing

Gene sequencing *or nucleotide sequencing*, and phylogenetic analysis of isolated influenza A viruses has been a source of data from where researchers have hypothesized the origins and sources of the components of the viral RNA sequences. Yamamoto, et al. (2011) investigated fecal samples collected from Siberian migratory waterfowl for the presence of influenza viral H5 HA and N1 NA genes. Nulceotide sequencing was performed on samples positive for H5 HA and N1 NA genes (Yamamoto, et al., 2011). The authors of the Yamamoto, et al. study concluded the results of nucleotide sequencing indicate highly pathogenic avian influenza H5N1 virus had not become dominant in the nesting lakes of Siberia prior to this study.

As aforementioned in chapter 2, Zhang et al. (2011) collected 200 ml water samples from areas near the habitat of migratory birds in East Dongting Lake, Yueyang City, Hunan Province for laboratory analysis. The phylogenic analysis of the isolated H10N8 virus indicated all the eight gene segments were of aquatic avian origin and belonged to a Eurasian lineage (Zhang et al., 2011). The authors of the Zhang et al. study concluded the H10N8 subtypes of avian viruses might pose a potential threat to mammals

and multiple amino acid substitutions are liklely to be involved in the adaptation of H10N8 influenza virus to mice.

As previously detailed in chapter 2, Fouchier, et al. (2005) isolated influenza A viruses from black-headed gill (*Larus ridibundus*) in Sweden. These isolated influenza A viruses could not be classified to the 15 known HA subtypes. The authors hypothesized the HA of the black-headed gull viruses represent a new HA subtype (Fouchier, et al., 2005). The researchers of the Fouchier, et al. study applied nucleotide sequence analysis, phylogenic analysis, and double immunodiffusion assay methods to the 10 samples from black-headed gulls. The results of the assay methods were further analyzed with BLAST available from GenBank (Fouchier, et al., 2005). Fouchier, et al. asserted the results provide strong evidence for the classification of the HA of the Swedish black-headed gull virus as H16.

The review of the literature about the topic of the current avian influenza A (H5N1) pandemic revealed the seriousness and global concern as a major pandemic threat to humans. As of date, the current WHO phase of pandemic alert for avian influenza A (H5N1) is: ALERT (World Health Organization, 2013). The potential for further spread of avian influenza A (H5N1) virus and the high case fatality rate warrants further surveillance. For these reasons, I investigated the burden of avian influenza A (H5N1) in recirculating artificial ponds in rural and urban geographical locations.

For this dissertation study, I utilized Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California) for influenza A virus (H5N1) gene sequencing. Water samples positive for hemagglutination properties of influenza A

viruses on MDCK cells and positive for influenza A virus by real time RT-PCR analytical phase were submitted for H5N1 gene sequencing . All other samples were excluded. All pertinent data were recorded in the laboratory notebook. The results of the avian influenza A (H5N1) gene sequencing were compared to the avian influenza A (H5N1) database repository at GenBank.

Data Analysis Plan

In this dissertation study, I upheld society's trust that scientific research results are an honest and accurate reflection of a researcher's work (National Academy of Sciences, National Academy of Engineering, and Institute of Medicine of the National Academies, 2010). This manuscript includes an outline for a data analysis plan examining the burden of influenza A virus in recirculating artificial ponds in rural and urban geographical locations. The primary data analysis were a cross-sectional approach comparing proportions, bivariate and multivariate logistic regression analysis of recirculating artificial ponds in rural and urban geographical locations. The independent variables included: geographic community location (rural or urban), GPS location (latitude and longitude), altitude, approximate water pond surface area. The dependent variables included: influenza A virus detection, influenza A virus infectivity endpoint titer, influenza A virus (H5N1) characterization. Variables evaluated as possible mediators and moderators included: water pH at collection, water salinity at collection, water temperature at collection, and presence or absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*).

The statistical analysis software was IBM SPSS Statistics 21 and Microsoft Office Excel 2007. Geographical graphing and mapping software were Google Earth. Data integrity was maintained by recording data and observations with ink into field notebook and laboratory notebook. Hand written entries were transferred or transcribed to Microsoft Office Excel 2007 Spreadsheets. All electronic data had digital backup and was password protected. Since I collected primary data, data cleaning and screening procedures were not necessary.

Table 1

Analysis Matrix for Influenza A Virus in Ponds Study

Study objective	Concept	Data source	Level of measurement	Analysis procedure
1.1	Proportion and probability of influenza A virus in geographical locations	Pond water analysis by real time RT-PCR	Categorical - Quantitative	Proportions, relative risk, probabilities, odds, and odds ratio
1.2	Infectivity influenza A virus in geographical locations	Pond water analysis by infectivity assay	Categorical, Interval scale (titer) - Quantitative	Frequencies, means, percent description
2.1	Regression analysis of factors contributing to influenza A virus presence	Pond water analysis by real time RT-PCR, field research data	Categorical, interval - Quantitative	Multiple logistic regression
2.2	Geospatial trending of influenza A virus	Field research data (GPS), Google Earth mapping	Nominal - Quantitative	Mapping, frequencies, means, percent description
3	Regression analysis of factors contributing to influenza A virus infectivity	Pond water analysis by infectivity assay, field research data	Interval scale - Quantitative	Multiple linear regression
4	H5N1 gene sequencing and identification	Pond water analysis by real time RT-PCR, gene sequencing by commercial laboratory	Categorical - Quantitative	Frequencies, means, percent description

Research Question Analysis

In this study the proportion and probability of the presence of influenza A virus were investigated in recirculating artificial water ponds in rural and urban geographical locations. Rural ponds were viewed as one population and urban ponds as another population. To approach *(R1)*, the underlying test statistics were binomial distribution. The binomial distribution refers to random variables with two outcomes (Forthofer et al., 2007). For the research question *(R1)*, the outcome variables were influenza A virus detection, influenza A virus infectivity endpoint titer, and influenza A virus (H5N1) characterization. The independent variables were the geographical location of the recirculating artificial water pond sampling: rural or urban community. Independent variables were coded [0 = rural community, 1 = urban community].

The statistical analysis examined the difference between the two proportions (rural and urban communities), the relative risk, probabilities, odds, and odds ratio of influenza A virus detection in rural and urban community recirculating artificial water ponds. Interpretation of the findings included 95% confidence intervals (CIs) for difference between the two proportions, relative risk, and odds ratio. Influenza A virus detection were coded [0 = negative, 1 = positive]. The statistical analyses also included the binomial distribution of the dependent ordinal variables: influenza A virus infectivity endpoint titer, and influenza A virus (H5N1) characterization. For the statistical analysis, the results of the hemagglutination titration levels-for all samples (+) positive for the influenza A virus M gene sequence-were transformed to integer values using the binary logarithm [$\log_2(n)$] (i.e. titration level 1:16 = $\log_2(16) = 4$). To account for negative

hemagglutination (integer transformation = 0), the value of 1 is added to each integer value. Thus, the mathematical transformation is of the form: $\log_2 (n) + 1$ (i.e. titration level 1:16 = $\log_2 (16) + 1 = 5$).

To approach *(R2)*, the underlying test statistic was logistic regression. Logistic regression is a method for examining the relationship between a dependent variable with two levels and one or more independent variables. Forward method stepwise logistic regression was used to test *(R2)*. Field (2009) asserted that stepwise logistic regression to be more appropriate for research situations where no previous research exists. Presently, no known research is underway to investigate and compare the proportion of influenza A virus in artificial rural and urban ponds. Additionally, Field asserted that forward stepwise method is more likely to exclude predictors involved in suppressor effects. However, the forward stepwise method runs a higher risk of making a Type II error. For this research question, Type II error is favorable over a Type I error. A Type I error may significantly mislead future research, whereas a Type II error may have less influence. To evaluate how the model overall fits the data, the likelihood ratio test (Sig. $p< 0.05$) , the Wald statistic, and the odds ratio [Exp(B)] were included in the analysis.

For research question *(R2)*, the dependent variable was *influenza A virus detection*. Influenza A virus detection were coded [0 = negative, 1 = positive]. The independent variables were the geographical location of the recirculating artificial water pond sampling (rural or urban community), latitude and longitudinal coordinates, altitude, and estimate surface area, and presence or absence of waterfowl (*Anseriformes*) or shorebirds (*Charadriiformes*) at the time of sample collection. Independent variable

geographical location were coded [0 = rural community, 1 = urban community]. The independent variables *latitudinal coordinates, longitudinal coordinates,* and *estimate surface area were* separate continuous variables. The independent variable *altitude* were transformed into 500 ft. elevation increments. The independent variable *presence or absence of waterfowl or shorebirds* were coded [0 = not present, 1 = present].

Google Earth mapping software were utilized in attempt to identify geospatial trending of positive and negative influenza A virus samples. Global positioning system (GPS) location of samples were uploaded to Google Earth as waypoints. The waypoints were identified by sample number. A multicolor-code system were used to indicate influenza A virus presence for each sample number. A map of sample collection across California were produced using this data. Mapping of the influenza A virus status of each sample number is a method of geospatial trending of influenza A virus distribution across California.

To approach (R3), multiple linear regression analysis was used. Multiple linear regression is a method for examining the relation between one normally distributed dependent variable and more than one continuous independent variable (Forthofer et al., 2007). Interpretation of the findings included the *t*-statistics test. If the significance of the *t*-statistic is less than 0.05, then it were assumed that *b* is significantly different from 0, therefore the predictor makes a significant contribution to predicting the outcome (Field, 2009). For research question (R3), the dependent ordinal variable was influenza A virus infectivity endpoint titer. For the statistical analysis, the results of the hemagglutination titration levels-for all samples (+) positive for the influenza A virus M gene sequence-

were transformed to integer values using the binary logarithm [$\log_2(n)$] (i.e. titration level 1:16 = $\log_2(16) = 4$). To account for negative hemagglutination (integer transformation = 0), the value of 1 is added to each integer value. Thus, the mathematical transformation is of the form: $\log_2(n) + 1$ (i.e. titration level 1:16 = $\log_2(16) + 1 = 5$). The independent continuous variables were *water temperature, water pH,* and *water salinity*. The data were stratified by geographical location. *Geographical location* were coded [0 = rural community, 1 = urban community].

To approach (R4), samples positive for influenza A virus (H5N1) by real-time RT-PCR were submitted for gene sequencing analysis to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California). The influenza A virus (H5N1) gene sequencing results were compared to the influenza A viral gene sequence database GenBank (National Institute of Health genetic sequence database).

Threats to Validity

There are two threats to the external validity of this study. The first threat to external validity is centered to the theoretical framework of this dissertation. The theoretical framework for this study were based on the hypotheses proposed by Webster et al. (1992) and others that (a) migratory waterfowl are the natural reserve of influenza viruses, and (b) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. The geographical area was the state boundaries of California. Thus, the findings of this dissertation are limited to geographical locations along the

migratory flyways of the waterfowl within the boundaries of California. To address this threat to the external validity of this dissertation study, the sampling and data analysis plan were presented in a fashion so the study may be reproduced accurately by others using the same or different geographical locations.

The second threat to external validity is a result of the inclusion criteria. Recirculating artificial water ponds require mechanical equipment for recirculation. This study does not include the type or design of the mechanical equipment used for recirculation as an independent variable. Thus, the mechanical equipment used for recirculation cannot be evaluated as a mediator or moderator of the outcome variables of this dissertation study. However, estimated surface area of the recirculating artificial water ponds as an independent variable may be of significant interest to future studies seeking to investigate factors that associate to the burden of influenza A virus at the human-animal interface in communities.

The primary data collection of dissertation study is heavily weighted by laboratory instrumentation and molecular analysis. There are two threats to the internal validity of this dissertation study: instrumentation and laboratory assays. Firstly, inaccuracy of the instrument used for this study may systematically alter the data. Secondly, inaccuracy of the laboratory assays may fail to accurately detect influenza A virus. To address the possible inaccuracy of the instrumentation, all samples will use the same instrumentation. Thus, errors, if identified, may be corrected across all samples equally. To address the possible inaccuracy of the laboratory assays, positive and negative control samples were processed simultaneously as study samples. The positive

and negative control influenza A virus samples have not been determined. Threats to statistical conclusion validity have not been identified.

Ethical Procedures

This dissertation study was conducted in an ethical manner. I submitted an application to the Walden University IRB for approval to conduct research and I did not collect any data until Walden University IRB approval was received. The IRB approval number for this dissertation research study is: 04-16-14-0293110. The populations were artificial recirculating water ponds in the geographic locations of rural and urban communities in California. Since some of the samples were under public or private ownership, a signed Collection Site Contact Letter was offered and Letter of Cooperation was received before any field analysis or water collection were conducted (see Appendices A and B for templates of letters). Environmental water sampling underscored the research design of this study. Therefore, the research adhered to the Code of Ethics and Standards of Practice for Environmental Professionals. The objectives of Environmental Professionals are to conduct their personal and professional lives and activities in an ethical manner (National Association of Environmental Professionals, n.d). The Code of Ethics and Standards of Practice for Environmental Professionals are: Creed

The objectives of an Environmental Professional are:

1. To recognize and attempt to reconcile societal and individual human needs with responsibility for physical, natural, and cultural systems.
2. To promote and develop policies, plans, activities and projects that achieve

complementary and mutual support between natural and man-made, and present and future components of the physical, natural and cultural environment.

Ethics

As an Environmental Professional I will:

1. Be personally responsible for the validity of all data collected, analyses performed, or plans developed by me or under my direction. I was responsible and ethical in my professional activities.
2. Encourage research, planning, design, management and review of activities in a scientifically and technically objective manner. I will incorporate the best principles of the environmental sciences for the mitigation of environmental harm and enhancement of environmental quality.
3. Not condone misrepresentation of work I have performed or that was performed under my direction.
4. Examine all of my relationships or actions, which could be legitimately interpreted as a conflict of interest by clients, officials, the public or peers. In any instance where I have financial or personal interest in the activities with which they are directly or indirectly involved, I will make a full disclosure of that interest to my employer, client, or other affected parties.
5. Not engage in conduct involving dishonesty, fraud, deceit, or misrepresentation or discrimination.
6. Not accept fees wholly or partially contingent on the client's desired result

where that desired result conflicts with my professional judgment.

Guidance for Practice as an Environmental Professional

As an Environmental Professional I will:

1. Encourage environmental planning to begin in the earliest stages of project conceptualization.
2. Recognize that total environmental management involves the consideration of all environmental factors including: technical, economical, ecological, and sociopolitical and their relationships.
3. Incorporate the best principle of design and environmental planning when recommending measures to reduce environmental harm and enhance environmental quality.
4. Conduct my analysis, planning, design and review my activities primarily in subject areas for which I am qualified, and shall encourage and recognize that participation of other professionals in subject areas where I am less experienced. I shall utilize and participate in interdisciplinary teams wherever practical to determine impacts, define and evaluate all reasonable alternatives to proposed actions, and assess short-term versus long-term productivity with and without the project or action.
5. Seek common, adequate, and sound technical grounds for communication with and respect for the contributions of other professionals in developing and reviewing policies, plans, activities and projects.
6. Determine the policies, plans, activities or projects in which I am involved are

consistent with all governing laws, ordinances, guidelines, plans and policies to the best of my knowledge and ability.

7. Encourage public participation at the earliest feasible time in an open and productive atmosphere.
8. Conduct my professional activities in a manner that ensures consideration of technically and economically feasible alternatives.

Encourage Development of the Profession

As an Environmental Professional I will:

1. Assist in maintaining the integrity and competence of my profession.
2. Encourage education and research and the development of useful technical information relating to the environmental field.
3. Be prohibited from lobbying in the name of the National Association of Environmental Professionals.

Advertise and present my services in a manner that avoids the use of material and methods that may bring discredit to the profession.

The data collected were confidential. The statistical analysis software was IBM SPSS Statistics 21 and Microsoft Office Excel 2007. Geographical graphing and mapping software were Google Earth. Data integrity was maintained by recording data and observations with ink into field notebook and laboratory notebook. Hand written entries were transferred or transcribed to Microsoft Office Excel 2007 Spreadsheets. All electronic data had digital backup and was password protected. Since I collected primary data, data cleaning and screening procedures were not necessary. The raw data will be

retained for no less than 60 months and no longer than 120 months from completion of this dissertation study. At the end of the retention period, the field notebooks, laboratory notebooks, digital data, and digital backup data will be destroyed or deleted.

I claim no conflict of interest. The Biological Safety Level 2 laboratory where sample preparation, hemagglutination, and real-time RT-PCR assays were performed is located at the California State University Channel Islands, Camarillo CA campus. I am employed as a paid part-time lecturer at this University.

Summary

This baseline study of the proportion of influenza A virus in urban and rural community settings resulted in knowledge and biological data that may be of significant interest at the human-animal interface. Artificial water ponds in rural and urban communities are sites of the human-animal interface. Several studies investigated the presence or persistence of influenza viruses to natural waterfowl wetlands (Austin & Hinshaw,1984; Ferro, et al.,2010; Lang et al., 2008). The purpose of this quantitative study was to extend the previous environmental virology research of influenza in natural waterfowl wetlands to rural and urban communities. The dissertation study method was quantitative using a cross-sectional design. In this quantitative dissertation study, I collected and analyzed primary data. A convenience sampling approach was used. The geographical area was the state boundaries of California. Equal sample sizes from rural and urban Californian communities were attempted.

The methodology for characterization of influenza viruses has shown similarity across the literature. The methodologies applied in this dissertation are aligned to the

published literature. These methodologies include: virus detection by RT-PCR (Poddar et al., 2002), infectivity studies using MDCK cell line (Brown et al., 2009), nucleotide sequencing of the RNA genome (Yamamoto, et al., 2011), and phylogenic analysis using BLAST available from GenBank (Fouchier, et al., 2005).

The primary data analysis was a cross-sectional approach to compare proportions, and bivariate and multivariate regression analysis of samples from recirculating artificial ponds in rural and urban geographical locations. The independent variables included: geographic community location (rural or urban), GPS location (latitude and longitude), altitude, approximate water pond surface area. The dependent variables included: influenza A virus detection, influenza A virus infectivity endpoint titer, influenza A virus (H5N1) characterization. Variables evaluated as possible mediators and moderators included: water pH at collection, water salinity at collection, water temperature at collection, and presence or absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*).

The results of the data analysis were presented in table format, APA format interpretation, graphs, and maps. The organization of the results of data analysis is aligned to the research questions. The results of the data analysis may be of significant interest to the scientific community.

Chapter 4: Results

The “Spanish” influenza H1N1 pandemic of 1918–1919, which caused approximately 50 million deaths worldwide, remains an ominous warning to public health (Taubenberger & Morens, 2006). Since then, new subtypes of human influenza A viruses have been detected at various times: in 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, the H3N2 subtype (Hong Kong virus) appeared; and in 1977, the H1N1 virus reappeared (Webster et al., 1992). Influenza disease emergence data are collected year-round, but economic strain on global public health to prevent and treat human influenza outbreaks is enormous. Therefore, it is imperative to identify potential sources of the virus to help minimize outbreak occurrence.

There are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. Gaps in understanding the role of the physical and biogeochemical environment as an integral part of the influenza A viral transmission also exist (Lang et al., 2008). More importantly, gaps in knowledge about the burden of influenza A virus in rural and urban community settings remain present. IRAT is expected to prompt additional studies to address these key gaps in the knowledge (Trock et al., 2012).

The problem in this study was that public health scientists have been battling emerging human influenza diseases with tactile and reactionary methods because there was a lack of knowledge and data at the human-animal interface. This baseline study of the proportion of influenza A virus in urban and rural community settings may provide

knowledge and biological data of significant interest at the human-animal interface. This biological data may be of significant interest for the development of IRAT.

Research Design and Rationale

Presently, no known research is underway to investigate and compare the proportion of influenza A virus in recirculating artificial ponds in rural and urban geographical locations. A review of current literature did not identify any other studies that attempted to collect influenza A virus data from artificial water ponds in rural or urban communities. Several studies investigated the presence or persistence of influenza viruses to natural waterfowl wetlands (Austin & Hinshaw,1984; Ferro, et al.,2010; Lang et al., 2008). The purpose of this quantitative study is to extend the previous environmental virology research of influenza in natural waterfowl wetlands to rural and urban communities.

For this quantitative dissertation study, I collected and analyzed primary data. The epidemiological triangle provided the framework for the primary data collected. The epidemiological triangle has three vertices: host, agent, and environment. The host is the organism harboring the disease. For influenza disease, the reservoir hosts are migratory waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*; Franklin et al., 2011). The agents were the influenza A viruses. The environments were the natural and artificial habitats where the hosts are found.

The independent environmental variables of this study were the geographical locations of the artificial water ponds and geochemical properties of the artificial water pond samples. The geographical location data included rural or urban community, latitude

and longitudinal coordinates, altitude, and approximate water pond surface area. The geochemical properties of the artificial water pond sample measure included pH, salinity, and temperature. Additionally, the presence or absence of waterfowl at the time of sample collection was included as an independent host variable. The dependent agent variables were influenza A viruses. Influenza A virus data included detection for M gene by real time RT-PCR using World Health Organization recommended primer sequences, infectivity by hemagglutination assay using MDCK cell line, and nucleotide sequencing for H5N1 influenza A virus using World Health Organization recommended primer sequences at Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California).

The human-animal interface is recognized as a complex but critical juncture to which new paradigms are emerging (World Health Organization, 2011). The aquatic virus reservoir is a human-animal interface. Franklin et al. (2011) suggested aquatic virus reservoirs give rise to indirect transmission, which would alter the transmission dynamics, beyond just direct interactions between infectious and susceptible individuals. The quantitative research design of this dissertation was intended to investigate aquatic virus reservoirs as a human-animal interface. Further, the quantitative design of this dissertation addressed the following research questions.

Research Questions

R1: Is there a difference in the burden of influenza A virus in rural ponds compared to urban ponds?

$H_0$1: The burden of influenza A virus in community ponds has no association to geographical location.

H_a1: The burden of influenza A virus in community ponds is associated to geographical location.

In this study the proportion and probability of the presence of influenza A virus was investigated in recirculating artificial ponds in rural and urban geographical locations. Rural ponds were viewed as one population and urban ponds as another population. The dependent agent variables were influenza A viruses.

R2: Is there an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds?

$H_0$2: There is no association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

H_a2: There is an association between geographic location, altitude, estimated surface area and observed presence of waterfowl, and the proportion of influenza A virus in rural ponds compared to urban ponds.

In this study, rural community ponds were viewed as one population and urban community ponds as another population. The comparison of these two populations included proportion of influenza A virus(s), latitude and longitudinal coordinates, altitude, and approximate water pond surface area, and the presence or absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) at the time of sample

collection. These factors were included as an independent environment and host variables. The dependent agent variables are influenza A viruses.

R3: Is there an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds?

$H_0$3: There is no association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

H_a3: There is an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds.

In this dissertation study, I investigated if the findings of the Brown et al. (2009) study were consistent in rural and urban community pond samples. The geochemical properties of the artificial water pond sample measure included pH, salinity, and temperature. The dependent agent variable was infectivity of influenza A viruses.

R4: Are H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds novel nucleotide sequences or have the nucleotide sequences been previously identified elsewhere?

$H_0$4: The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are not novel nucleotide sequences and the nucleotide sequences have been previously identified elsewhere.

H_a4: The H5N1 influenza A viral subtypes detected in the sample of rural and urban ponds are novel nucleotide sequences and the nucleotide sequences have not been previously identified elsewhere.

In this study, water samples were collected directly from rural and urban ponds. World Health Organization recommended real time RT-PCR primers were used for influenza A virus H5N1 nucleotide sequencing. The laboratory analysis for the presence of influenza A virus and influenza A virus infectivity were conducted in a biosafety level 2 laboratory at the California State University Channel Islands campus. The analysis for the presence of influenza A virus used commercially available influenza A/B viral real time RT-PCR detection kits suitable for water analysis. Water samples positive for H5N1 influenza A viral strains were submitted for nucleotide sequencing analysis to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California). The H5N1 influenza A virus nucleotide sequencing results were compared to the influenza A viral gene sequence database GenBank (National Institute of Health genetic sequence database) using the National Center for Biotechnology Information (NCBI) BLAST.

Organization of the Chapter

This chapter is organized in a manner to present the data and research findings in a clear and understandable format. Tables and figures are included in this manuscript to provide the reader with a visual understanding of the data and data analysis. Following the above introductory sections, the constraints and discrepancies of the research are presented. This section is followed by the descriptive statistics of the data and then the analysis of the data pertaining to each research question. For the descriptive statistics section; the field research and laboratory research data analysis is presented in three subsections. The subsections begin with the overall statewide sample data, followed by the urban counties' sample data, and then the rural counties' sample data. Following the

descriptive statistics are the analyses of the field research and laboratory research data, as it pertains to each research question. These sections are followed by a chapter summary of the findings of this dissertation research study.

Data Collection

Time and Resource Constraints

Applied environmental studies can be classified as baseline, monitoring, or impact studies (Green, 1979). This dissertation is a baseline environmental study. Green (1979) asserted a *baseline study* is one in which data are collected and analyzed for the purpose of defining the present state of the biological community, the environment, or both (p. 68). In this dissertation study, I attempted to define the present state of the biological community of recirculating artificial ponds in rural and urban geographical locations in California. The temperate climate of California did not pose seasonal constraints. However, to define the present state of the biological community, a cross-sectional sampling approach was used. To limit threats to the internal validity of the study, pond water samples were collected over the shortest time period, as possible. Field research and water sample collection for this study were conducted from June 15, 2014 through August 18, 2014. The laboratory water sample processing and analysis for this study were conducted from June 17, 2014 through September 4, 2014. There were no resource contraints apparent.

Discrepancies in Data Collection from the Planned

Unexpectedly, the weather and natural disasters in California had a negative effect on field research and water sample collection. As previously noted, field research and

water sample collection for this study were conducted from June 15, 2014 through August 18, 2014. I attempted to collect an equal sample size from rural and urban communities; a representative sampling from each of the 21 counties considered rural areas, and 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l). However, the severe drought conditions in California resulted in a limited the number of available artificial recirculating water ponds for sampling. Furthermore, the wildfires in Northern California during the time frame of the field research and data collection made several of the planned pond water locations unsafe for travel and field research. As shown in Table 1 only a representative sampling from 14 counties considered rural areas ($N_{Rural} = 82$), and 25 counties considered metropolitan and not rural ($N_{Urban} = 100$) in California by California Business and Professions Code Section 19986(l) was achieved. Discrepancies in data collection for this study were not only limited to field research and water sample collection. Discrepancies also occurred during the laboratory data collection process.

Table 2

Water Sample Collection by County

County name	Code Section 19986(l) designation	Number of water samples collected	County name	Code Section 19986(l) designation	Number of water samples collected
Alameda	URBAN	0	Orange	URBAN	3
Alpine	RURAL	0	Placer	URBAN	7
Amador	RURAL	2	Plumas	RURAL	5
Butte	URBAN	1	Riverside	URBAN	4
Calaveras	RURAL	1	Sacramento	URBAN	4
Colusa	RURAL	4	San Benito	URBAN	3
Contra Costa	URBAN	0	San Bernardino	URBAN	4
Del Norte	RURAL	0	San Diego	URBAN	2
El Dorado	URBAN	0	San Francisco	URBAN	0
Fresno	URBAN	4	San Joaquin	URBAN	8
Glenn	RURAL	2	San Luis Obispo	URBAN	3
Humboldt	RURAL	0	San Mateo	URBAN	0
Imperial	URBAN	5	Santa Barbara	URBAN	3
Inyo	RURAL	7	Santa Clara	URBAN	6
Kern	URBAN	3	Santa Cruz	URBAN	3
Kings	URBAN	2	Shasta	URBAN	0
Lake	RURAL	0	Sierra	RURAL	0
Lassen	RURAL	2	Siskiyou	RURAL	8
Los Angeles	URBAN	5	Solano	URBAN	4
Madera	URBAN	3	Sonoma	URBAN	0
Marin	URBAN	0	Stanislaus	URBAN	0
Mariposa	RURAL	1	Sutter	URBAN	0
Mendocino	RURAL	0	Tehama	RURAL	14
Merced	URBAN	0	Trinity	RURAL	0
Modoc	RURAL	13	Tulare	URBAN	2
Mono	RURAL	4	Tuolumne	RURAL	4
Monterey	URBAN	6	Ventura	URBAN	4
Napa	URBAN	0	Yolo	URBAN	9
Nevada	RURAL	15	Yuba	URBAN	1

Regulatory issues hampered the data collection processes in the laboratory. The laboratory water sample processing and analysis for this study were conducted from June 17, 2014 through September 4, 2014. Positive and negative influenza A virus controls were planned to be used during the real time RT-PCR assay targeting the M gene sequence of influenza A viral genome and also during the hemagglutination MDCK plaque assay to investigate influenza A virus infectivity. Regulatory policies prevented the acquisition of a positive influenza A virus control. A readily available sterile normal saline solution was used for a negative influenza A virus control. Since a positive influenza A virus control was not available for the real time RT-PCR M gene assay, a fluorophore endpoint evaluation was computed. The endpoint evaluation was computed using the Bio-Rad CFX96 Touch Real time PCR Detection System (Bio-Rad, Hercules, California) instrument software. Since a positive influenza A virus control was not available for the hemagglutination MDCK plaque assays, the interpretation of the hemagglutination was based on the negative control hemagglutination and therefore, the results are highly subjective. There were also unexpected delays performing the laboratory analysis of collected water samples.

It was planned that pond water samples be prepared and tested for the hemagglutination MDCK plaque assay within 72 hrs of sample collection. Due to delays in MDCK cell culture growth and other outside factors; the goal of testing all water samples (N = 182) within 72 hrs of collection was not achieved. The extended delays of testing the water samples may have an adverse effect on influenza A virus viability and infectivity. The processing delays of the water samples range from same day (< 24 hrs) to

16 days (384 hrs) [*M* = 7.08 days (170 hrs), *SD* = 4.957 days, and *Mdn* = 7.00 days (168 hrs)]. Due to the unexpected delays in preparation and testing for hemagglutination using MDCK cells the results may not be truly representative of influenza A virus viability and infectivity. A positive influenza A virus control provides the desired experimental outcome for comparison against the samples. Without a positive influenza A virus control this comparison is not possible. Therefore, the results of the hemagglutination MDCK plaque assay should be interpreted with caution. It had also been planned to use 96 well U-bottom titration plates for the hemagglutination MDCK plaque assay. The 96 well U-bottom titration plates were not available. Thus, 96 well Flat-bottom titration plates were utilized in place for the hemagglutination MDCK plaque assay.

Results

Descriptive Statistics of Pond Water Samples

In this study the proportion and probability of the presence of influenza A virus was investigated in recirculating artificial ponds in urban and rural locations. As shown in Figure 1 and listed in Table 1 artificial pond water samples were collected from 14 of the 21 counties considered rural areas, and from 25 of the 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l). Urban counties' pond water samples [$N_{Urban} = 100$] were collected from 25 of the 37 counties considered metropolitan and not rural in California by California Business and Professions Code Section 19986(l). Rural counties' pond water samples [$N_{Rural} = 82$] were collected from 14 of the 21 counties considered rural and not metropolitan in California by California Business and Professions Code Section 19986(l).

As previously noted, in this study the proportion and probability of the presence of influenza A virus was investigated in recirculating artificial ponds in urban and rural locations The artificial pond water sample data for statistical analysis was selected from the overall statewide sampling data recorded during the field research and laboratory assays of this dissertation study. Also as previously noted, all water samples were verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence.

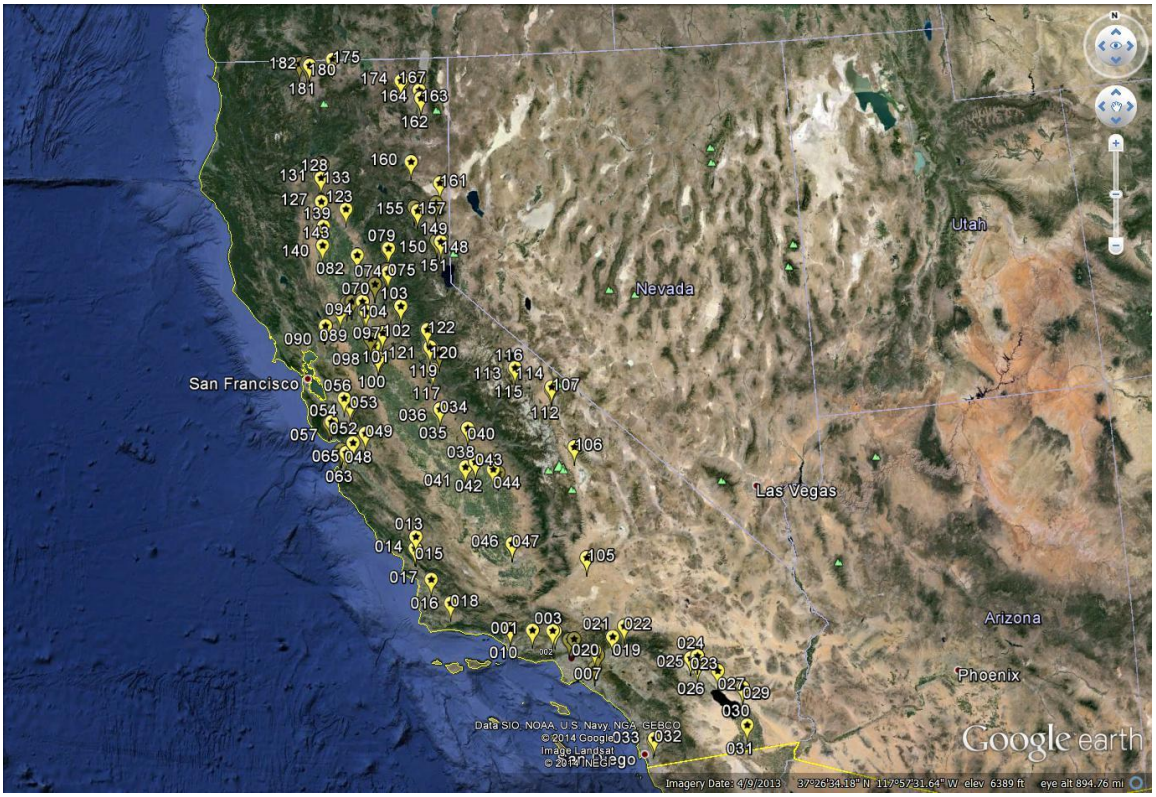

Figure 1. Data collection site map. Google Earth map of California showing collection site locations and sample numeration of pond water samples (*N* = 182).
Samples assayed by real time RT-PCR

Pond water samples were verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence. The real time RT-PCR M gene

assay was conducted using a Bio-Rad CFX96 Touch Real time PCR Detection System (Bio-Rad, Hercules, California) instrument and utilized two fluorescent dyes: FAM and HEX. The analysis software used was Bio-Rad CFX Manager 3.1. The software setting was: End cycles to average [5] and Percent of Range [10.0]. The results for the FAM fluorophore were: [Lowest RFU value = -3.71], [Highest RFU value = 3.41], [Negative Control Average = -0.453], and [Cut Off Value = -0.0668]. The number of statewide samples [N = 182] called (+) positive using FAM [N_{FAM} = 45, 45/182 = 24.7%]. The results for the HEX fluorophore were: [Lowest RFU value = -1.93], [Highest RFU value = 1.46], [Negative Control Average = 1.42], and [Cut Off Value = 1.42]. The number of statewide samples [N = 182] called (+) positive using HEX [N_{HEX} = 1, 1/182 = 0.5%]. The number of statewide samples [N = 182] verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence is [n = 45] (see Figure 2.). The confidence interval formula for a proportion is commonly known as: $CI = p \pm Z_{\alpha/2} \times \sqrt{[(p \times q) / n]}$, $(x, n - x \geq 5)$, where $p = x / n$, $q = 1 - p$, $\alpha = 1 -$ (Confidence Level/100) x = Frequency, n = Sample Size, and $Z_{\alpha/2}$ = Z-table value. Applying the data values of this study into the confidence interval formula the proportion of the overall statewide samples [N = 182] called (+) positive for the influenza A virus M gene sequence [n = 45] is P = 0.247, 95% CI [0.185, 0.310]. The effect size index Cohen's g is the departure from P = 0.50 (Cohen, 1988). Thus, the nondirectional calculation for Cohen's g is [$g = |P - 0.50|$]. For the overall statewide samples, the calculation of Cohen's g is [$g = |0.247 - 0.50| = 0.253$]; a large effect size (Cohen, 1988). Using G*Power 3.1.5, the post hoc computed Power = 0.999 (two tailed).

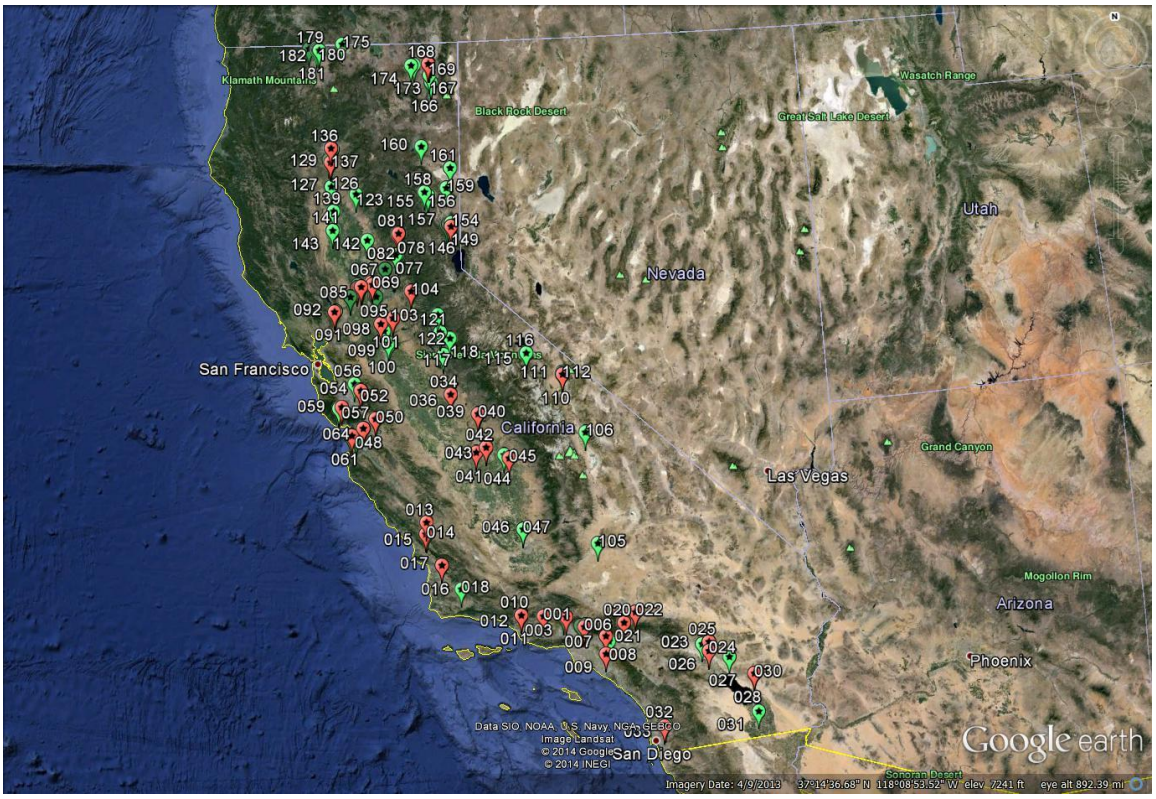

Figure 2. Statewide samples verified for their content of influenza A virus genome. Google Earth map of California showing statewide pond water samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence. [RED icon = (+) Positive for M gene sequence and GREEN icon = (-) Negative for M gene sequence].

Urban samples assayed by real time RT-PCR. The number of urban county samples [N_{Urban} = 100] called (+) positive using FAM [N_{FAM} = 36, 36/100 = 36.0%]. The number of samples [N_{Urban} = 100] called (+) positive using HEX [N_{HEX} = 1, 1/100 = 1.0%]. The number of urban county samples [N_{Urban} = 100] verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence is [n = 36] (see Figure 3). Applying the confidence interval formula for a proportion: $CI = p \pm Z_{\alpha/2} \times \sqrt{[(p \times q) / n]}$, $(x, n - x \geq 5)$, where $p = x / n$, $q = 1 - p$, $\alpha = 1 -$ (Confidence Level/100) x = Frequency, n = Sample Size, and $Z_{\alpha/2}$ = Z-table value, the proportion of the urban

counties' samples [N_{Urban} = 100] called (+) positive for the influenza A virus M gene sequence [n = 36] is P = 0.36, 95% CI [0.266, 0.454]. The effect size index Cohen's g is the departure from P = 0.50 (Cohen, 1988). Thus, the nondirectional calculation for Cohen's g is [g = |P – 0.50|]. For the urban counties' samples, the calculation of Cohen's g is [g = |0.36 – 0.50| = 0.14]; a medium effect size (Cohen, 1988). Using G*Power 3.1.5, the post hoc computed Power = 0.768 (two tailed).

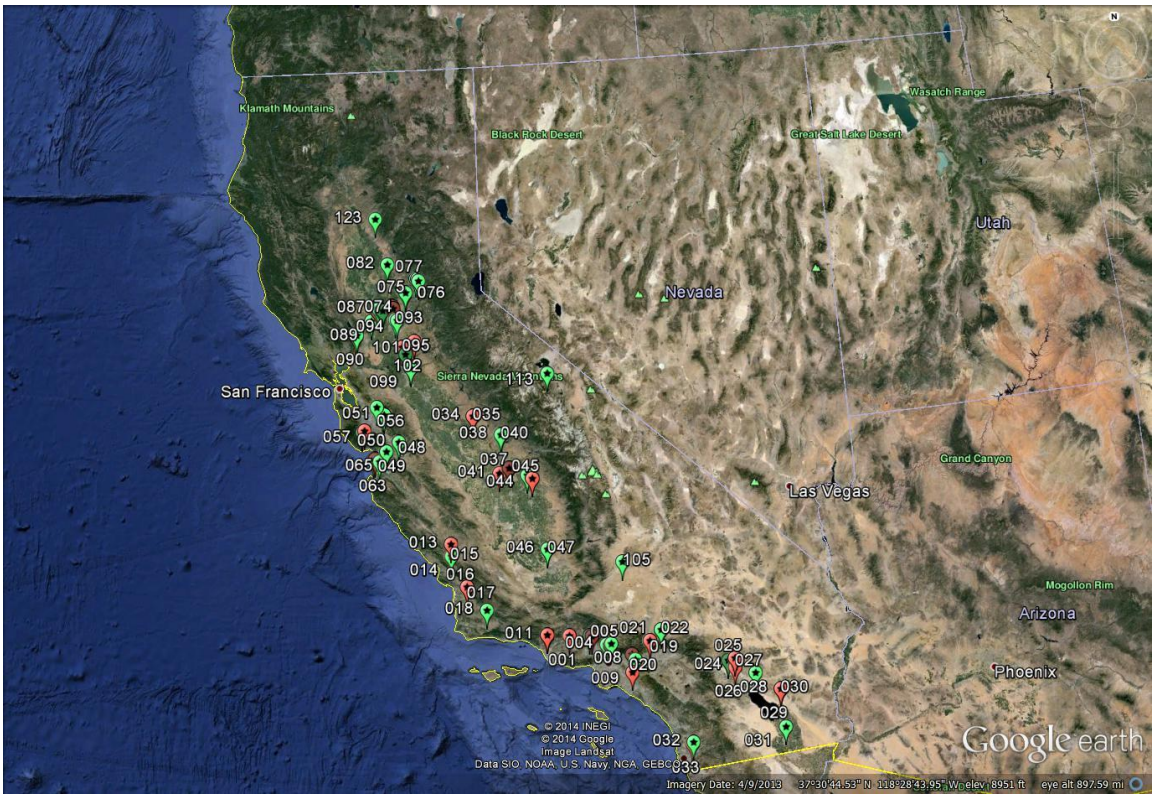

Figure 3. Urban county samples verified for their content of influenza A virus genome. Google Earth map of California showing urban counties' pond water samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence. [RED icon = (+) Positive for M gene sequence and GREEN icon = (-) Negative for M gene sequence].

Rural samples assayed by real time RT-PCR. The number of rural counties' samples [N_{Rural} = 82] called (+) positive using FAM [N_{FAM} = 7, 7/82 = 8.5%]. The

number of samples [N_{Rural} = 82] called (+) positive using HEX [N_{HEX} = 2, 2/82 = 2.4%]. The number of rural counties' samples [N_{Rural} = 82] verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence is [n = 9] (see Figure 4). Applying the confidence interval formula for a proportions: $CI = p \pm Z_{\alpha/2} \times \sqrt{[(p \times q) / n]}$, $(x, n - x \geq 5)$, where $p = x / n$, $q = 1 - p$, $\alpha = 1 - (\text{Confidence Level}/100)$ x = Frequency, n = Sample Size, and $Z_{\alpha/2}$ = Z-table value, the proportion of the rural counties' samples [N_{Rural} = 82] called (+) positive for the influenza A virus M gene sequence [n = 9] is $P = 0.11$, 95% CI [0.042, 0.177]. The effect size index Cohen's g is the departure from $P = 0.50$ (Cohen, 1988). Thus, the nondirectional calculation for Cohen's g is $[g = |P - 0.50|]$. For the rural counties' samples, the calculation of Cohen's g is $[g = |0.11 - 0.50| = 0.39]$; a large effect size (Cohen, 1988). Using G*Power 3.1.5, the post hoc computed Power = 1.000 (two tailed).

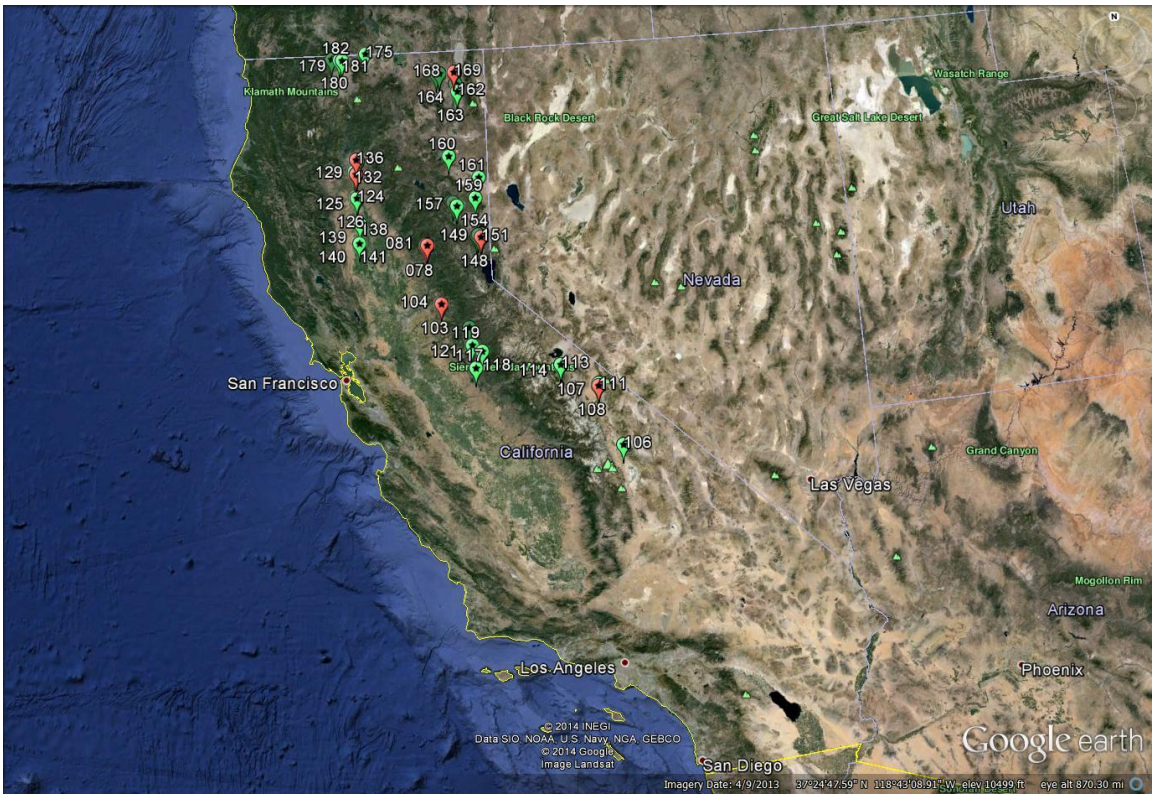

Figure 4. Rural county samples verified for their content of influenza A virus genome. Google Earth map of California showing rural counties' pond water samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence. [RED icon = (+) Positive for M gene sequence and GREEN icon = (-) Negative for M gene sequence].

Samples Assayed for Infectivity

Influenza A virus infectivity was tested using the hemagglutination MDCK plaque assay. Titrations were completed using a 96-well Flat-bottom plate. Serial dilutions across the 96-well Flat- bottom plate ranged from 1:1 to 1:2048. All statewide samples were assayed for influenza A virus infectivity. As shown in Table 2, the results of the overall statewide samples are [Neg = 79/182 (43.4%), 1:1 = 17/182 (9.3%), 1:2 = 16/182 (8.8%), 1:4 = 12/182 (6.6%), 1:8 = 15/182 (8.2%), 1:16 = 16/182 (8.8%), 1:32 =

9/182 (4.9%), 1:64 = 3/182 (1.6%), 1:128 = 7/182 (3.8%), 1:256 = 7/182 (3.8%), 1:512 = 0/182 (0.0%), 1:1024 = 0/182 (0.0%), 1:2048 = 1/182 (0.5%)].

Table 3

Results of Statewide Water Samples Assayed for Influenza A Virus Infectivity (N = 182)

Hemagglutination titer	Number of water samples	Percentage
Negative	79	43.41%
1:1	17	9.34%
1:2	16	8.79%
1:4	12	6.59%
1:8	15	8.24%
1:16	16	8.79%
1:32	9	4.95%
1:64	3	1.65%
1:128	7	3.85%
1:256	7	3.85%
1:512	1	0.55%
1:1024	0	0.00%
1:2048	1	0.55%

For the statistical analysis, only the data from those samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence were selected [n = 45]. All other statewide samples assessed as “positive for hemagglutination” are considered “false-positives” for influenza A virus infectivity using the hemagglutination MDCK plaque assay and are not included in the statistical analysis. The results of the overall statewide samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence are [Neg = 17/45 (37.8%), 1:1 = 6/45 (13.3%), 1:2 = 2/45 (4.4%), 1:4 = 5/45 (11.1%), 1:8 = 1/45 (2.2%), 1:16 =

4/45 (8.9%), 1:32 = 4/45 (8.9%), 1:64 = 1/45 (2.2%), 1:128 = 2/45 (4.4%), 1:256 = 3/45 (6.7%), 1:512 = 0/45 (0.0%), 1:1024 = 0/45 (0.0%), 1:2048 = 0/45 (0.0

Urban samples assayed for infectivity. All urban counties' samples were assayed for influenza A virus infectivity. As shown in Table 3, the results of the urban counties' samples are [Neg = 32/100 (32.0%), 1:1 = 8/100 (8.0%), 1:2 = 11/100 (11.0%), 1:4 = 4/100 (4.0%), 1:8 = 10/100 (10.0%), 1:16 = 10/100 (10.0%), 1:32 = 7/100 (7.0%), 1:64 = 3/100 (3.0%), 1:128 = 7/100 (7.0%), 1:256 = 7/100 (7.0%), 1:512 = 0/100 (0.0%), 1:1024 = 0/100 (0.0%), 1:2048 = 1/100 (1.0%)].

Table 4

Results of Urban Counties Water Samples Assayed for Influenza A Virus Infectivity (N = 100)

Hemagglutination titer	Number of water samples	Percentage
Negative	32	32.00%
1:1	8	8.00%
1:2	11	11.00%
1:4	4	4.00%
1:8	10	10.00%
1:16	10	10.00%
1:32	7	7.00%
1:64	3	3.00%
1:128	7	7.00%
1:256	7	7.00%
1:512	0	0.00%
1:1024	0	0.00%
1:2048	1	1.00%

For statistical analysis, only the data from those urban county samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene

sequence are utilized [n = 36]. All other urban counties samples assessed as "positive for hemagglutination" are considered "false-positives" for influenza A virus infectivity using the hemagglutination MDCK plaque assay and are not included in the statistical analysis. The results of the urban counties' samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence are [Neg = 13/36 (36.1%), 1:1 = 4/36 (11.1%), 1:2 = 2/36 (5.6%), 1:4 = 3/36 (8.3%), 1:8 = 0/36 (0.0%), 1:16 = 4/36 (11.1%), 1:32 = 4/36 (11.1%), 1:64 = 1/36 (2.8%), 1:128 = 2/36 (5.6%), 1:256 = 3/36 (8.3%), 1:512 = 0/36 (0.0%), 1:1024 = 0/36 (0.0%), 1:2048 = 0/36 (0.0%)].

Rural samples assayed for infectivity. All rural counties' samples were assayed for influenza A virus infectivity. As shown in Table 4, the results of the rural counties' samples are [Neg = 47/82 (57.3%), 1:1 = 9/82 (11.0%), 1:2 = 5/82 (6.1%), 1:4 = 8/82 (9.8%), 1:8 = 5/82 (6.1%), 1:16 = 6/82 (7.3%), 1:32 = 2/82 (2.4%), 1:64 = 0/82 (0.0%), 1:128 = 0/82 (0.0%), 1:256 = 0/82 (0.0%), 1:512 = 0/82 (0.0%), 1:1024 = 0/82 (0.0%), 1:2048 = 0/82 (0.0%)].

Table 5

Results of Rural Counties' Water Samples Assayed for Influenza A Virus Infectivity (N = 82)

Hemagglutination titer	Number of water samples	Percentage
Negative	47	57.30%
1:1	9	11.00%
1:2	5	6.10%
1:4	8	9.80%
1:8	5	6.10%
1:16	6	7.30%
1:32	2	2.40%
1:64	0	0.00%
1:128	0	0.00%
1:256	0	0.00%
1:512	0	0.00%
1:1024	0	0.00%
1:2048	0	0.00%

For statistical analysis, only the data from those rural counties' samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence are utilized [n = 9]**.** All other rural counties samples assessed as "positive for hemagglutination" are considered "false-positives" for influenza A virus infectivity using the hemagglutination MDCK plaque assay and are not included in the statistical analysis. The results of the rural counties' samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence are [Neg = 4/9 (44.4%), 1:1 = 0/9 (0.0%), 1:2 = 2/9 (22.2%), 1:4 = 2/9 (22.2%), 1:8 = 1/9 (11.1%), 1:16 = 0/9 (0.0%), 1:32 = 0/9 (0.0%), 1:64 = 0/9 (0.0%), 1:128 = 0/9 (0.0%), 1:256 = 0/9 (0.0%), 1:512 = 0/9 (0.0%), 1:1024 = 0/9 (0.0%), 1:2048 = 0/9 (0.0%)].%)]. The geographical location

data of the overall statewide samples was collected during the field research phase of the data collection.

Geographical Locations of Ponds Sampled

As previously noted, pond water samples were collected from 39 of the 58 counties in California. Latitude and longitudinal coordinate data were collected using a GARMIN® eTrex model 20 (Garmin, Ltd.) handheld GPS device. The coordinate data were converted to decimal values using the Federal Communication Commission Degrees, Minutes, Seconds and Decimal Degrees Latitude/Longitude Conversions online calculator (Federal Communications Commission, n.d.). The overall statewide samples were collected within the latitude range of [32° 39' 42.2202" N (32.661728)] to [41° 45' 56.7102" N (41.765753)] and within the longitudinal range of [115° 32' 41.9886" W (115.544997)] to [122° 39' 14.7708" W (122.654103)]. The urban counties' samples were collected within the latitude range of [32° 39' 42.2202" N (32.661728)] to [39° 45' 27.5286" N (39.757647)] and within the longitudinal range of [115° 32' 41.9886" W (115.544997)] to [122° 8' 32.7084" W (122.142419)]. The rural counties' samples were collected within the latitude range of [36° 34' 41.8686" N (36.578297)] to [41° 45' 56.7102" N (41.765753)] and within the longitudinal range of [118° 3' 54.3204" W (118.065089)] to [122° 39' 14.7708" W (122.654103)].

Altitude of Ponds Sampled

Altitude sample data were also collected using the same GARMIN® eTrex model 20 (Garmin, Ltd.) handheld GPS device. The overall statewide samples were collected within the altitudinal range from -209 ft. to 7903 ft. [M = 1622 ft., SD = 2140; *Skewness*

= 1.23, *SE* = 0.180; *Kurtosis* = 0.322, *SE* = 0.358]. For the statistical analysis, the raw statewide sample altitude data were transformed into 500 ft. elevation increments. As shown in Figure 5 the frequency of the transformed statewide altitude data is [Below Sea Level = 9, < 500 ft. = 96, < 1000 ft. = 8, < 1500 ft. = 7, < 2000 ft. = 5, < 2500 ft. = 1, < 3000 ft. = 12, < 3500 ft. = 1, < 4000 ft. = 1, < 4500 ft. = 21, < 5000 ft. = 6, < 5500 ft. = 0, < 6000 ft. = 6, < 6500 ft. = 5, < 7000 ft. = 0, < 7500 ft. = 0, > 7500 ft. = 4].

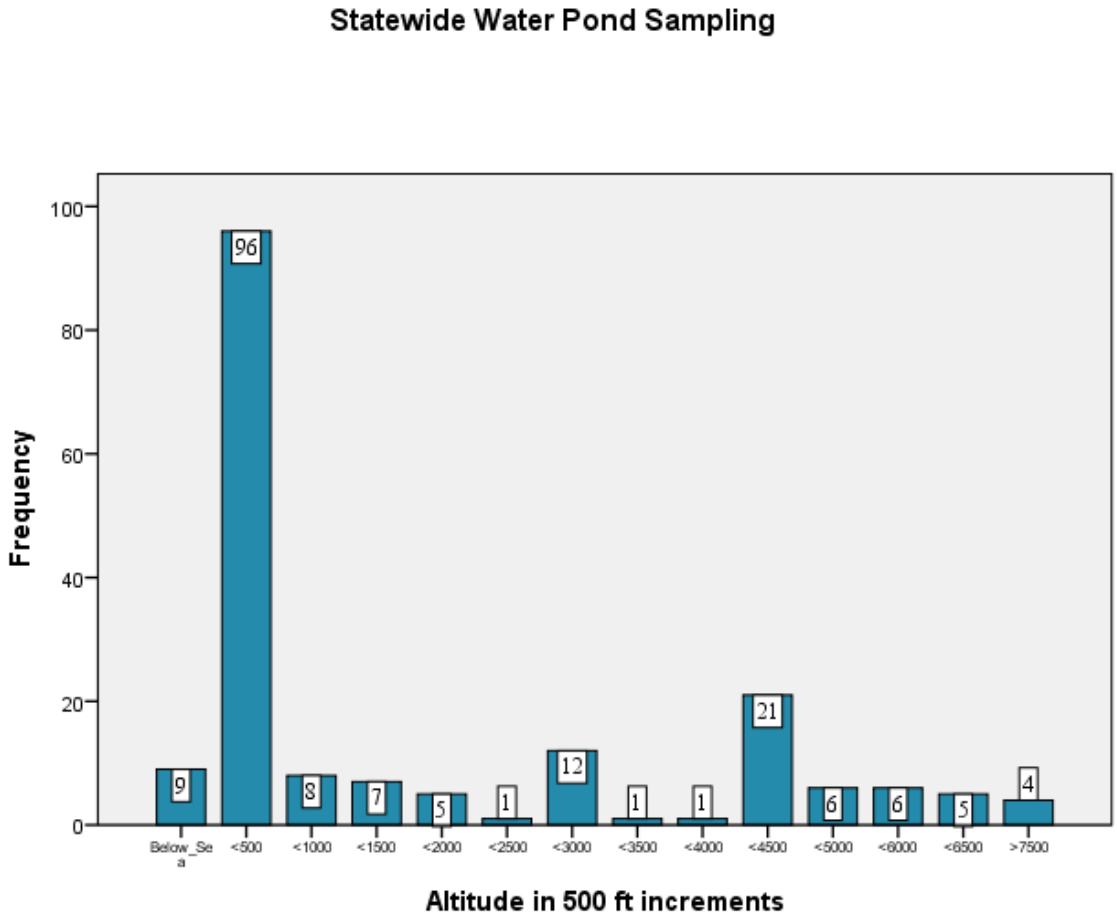

Figure 5. Frequency of statewide pond altitude data (elevation increment: 500 ft.).

Altitude of urban ponds sampled. The urban counties' samples were collected within the altitudinal range of -209 ft. to 2351 ft. [*M* = 280 ft., *SD* = 446; *Skewness* = 2.73, *SE* = 0.241; *Kurtosis* = 8.254, *SE* = 0.478]. For the statistical analysis, the raw urban counties' samples altitude data was transformed into 500 ft. elevation increments. As shown in Figure 6 the frequency of the transformed urban counties' samples altitude data is [Below Sea Level = 9, < 500 ft. = 76, < 1000 ft. = 5, < 1500 ft. = 4, < 2000 ft. = 2, < 2500 ft. = 1]. The approximate pond water surface area for urban counties' samples was selected from the overall statewide estimated pond water surface area data.

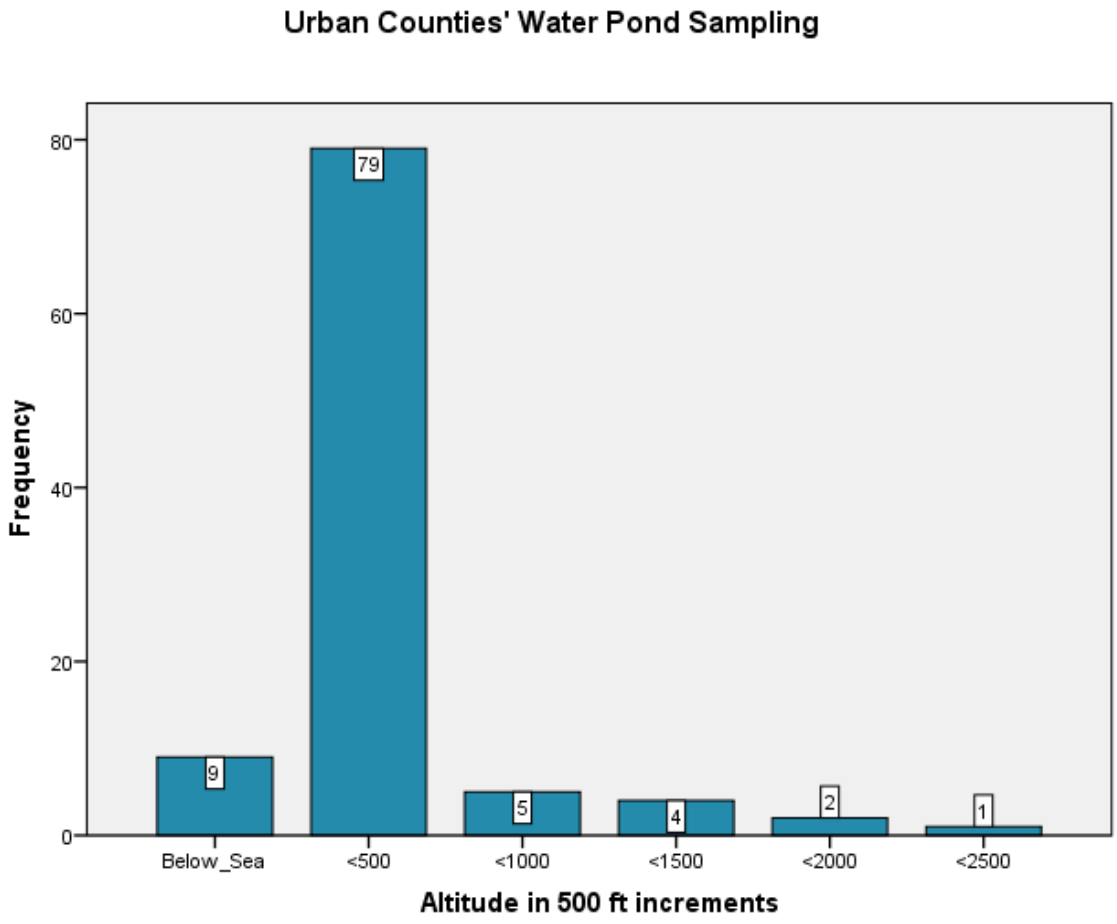

Figure 6. Frequency of urban counties' pond altitude data (elevation increment: 500 ft.).

Altitude of rural ponds sampled. The rural counties' samples were collected within the altitudinal range of 69 ft. to 7834 ft. [*M* = 3259, *SD* = 2249; *Skewness* = 0.093, *SE* = .266; *Kurtosis* = -0.921, *SE* = .526]. For the statistical analysis, the raw rural counties' samples altitude data was transformed into 500 ft. elevation increments. As shown in Figure 7 the frequency of the transformed rural counties' altitude data is [< 500 ft. = 17, < 1000 ft. = 3, < 1500 ft. = 3, < 2000 ft. = 3, < 2, < 3000 ft. = 12, < 3500 ft. = 1,

< 4000 ft. = 1, < 4500 ft. = 21, < 5000 ft. = 6, < 6000 ft. = 6, < 6500 ft. = 5, > 7500 ft. = 4].

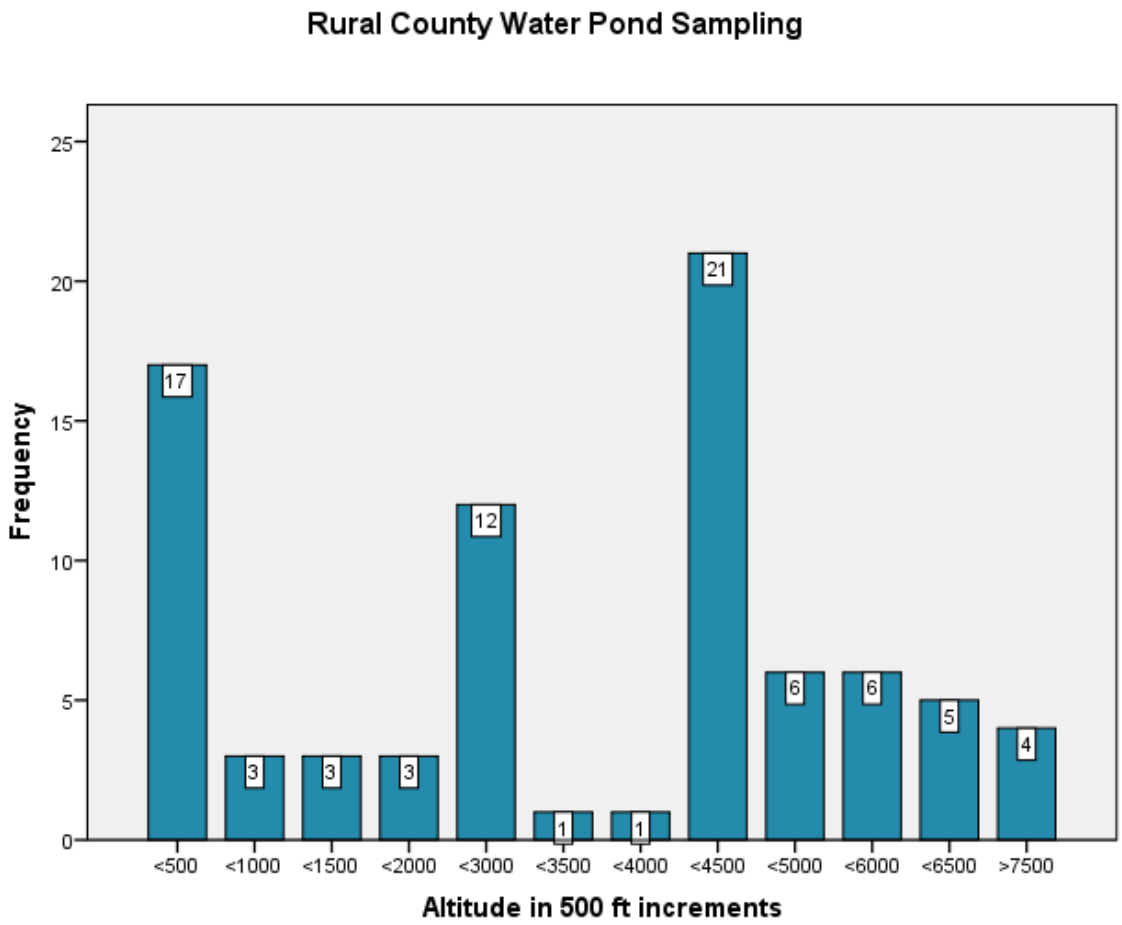

Figure 7. Frequency of rural counties' pond altitude data (elevation increment: 500 ft.).

Estimated Surface Area of Ponds Sampled

The approximate pond water surface area was estimated using either a Carl Zeiss Victory 8 x 26 T* PFR (Carl Zeiss, Sports Optics Gmbh, Germany) laser rangefinder for X-axis and Y-axis measurements, or the same GARMIN® eTrex model 20 (Garmin, Ltd.) handheld GPS device using the "Calculate Area" function, or Google Earth

estimated measurement function. The overall statewide samples were collected from artificial ponds with approximate surface areas ranging from 202 m^2 to 702408 m^2 [*M* = 22834 m^2, *SD* = 64023; *Mdn* = 5095 m^2; *Skewness* = 7.60, *SE* = 0.180; *Kurtosis* = 72.81, *SE* = 0.358]. For the statistical analysis, the raw overall statewide sample surface area data was transformed into 200 m^2 increments. The transformed statewide sample surface areas ranged from 1 to 3512 [*M* = 114, *SD* = 23.7; *Mdn* = 25; *Skewness* = 7.60, *SE* = 0.180; *Kurtosis* = 72.80, *SE* = 0.358] and is shown in Figure 8.

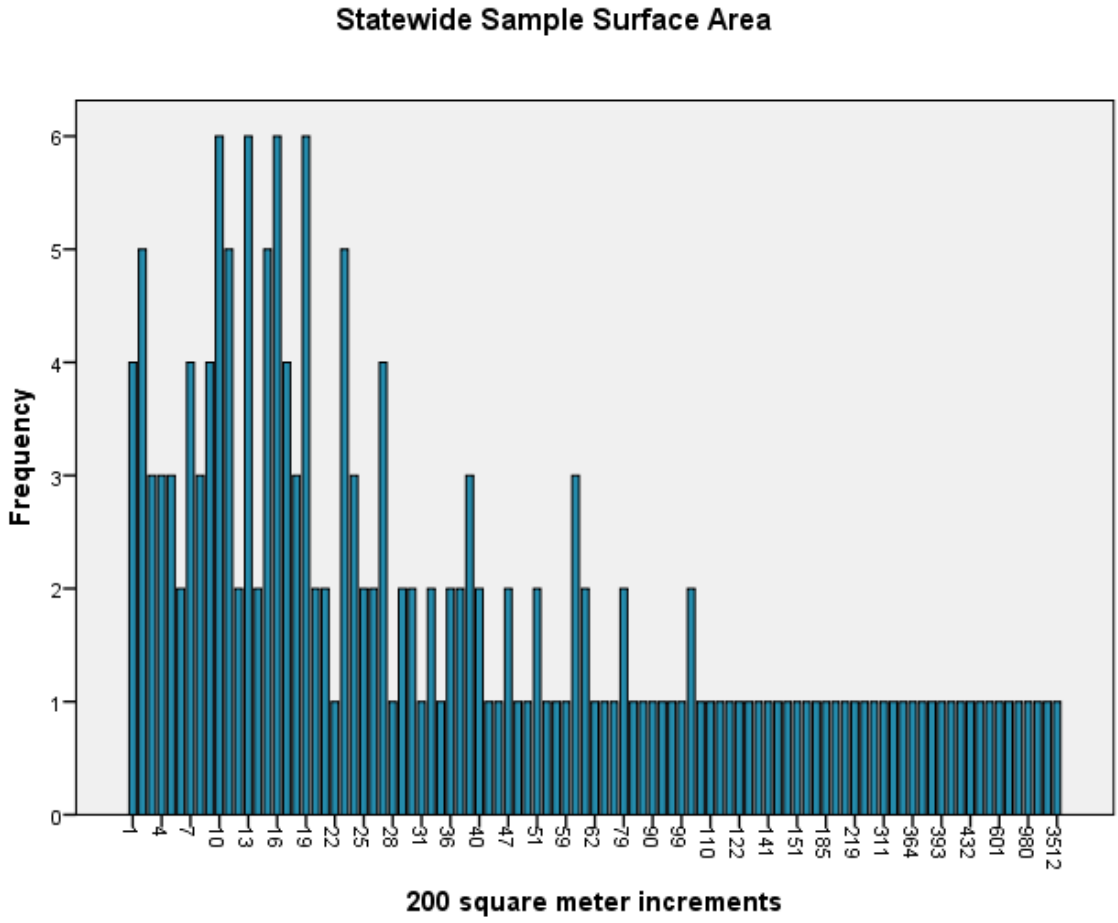

Figure 8. Frequency of statewide pond surface area data (surface area increment: 200 m2.).

Estimated surface area of urban ponds sampled. The urban counties' samples were collected from artificial ponds with estimated surface areas ranging from 327 m^2 to 702408 m^2 [*M* = 21617, *SD* = 72464; *Mdn* = 5733; *Skewness* = 8.611, *SE* = .241; *Kurtosis* = 80.611, *SE* = .478]. For the statistical analysis, the raw urban counties' sample surface area data was transformed into 200 m^2 increments. The transformed urban counties' samples surface areas range from 2 to 3512 [*M* = 108, *SD* = 362; *Mdn* = 29; *Skewness* = 8.612, *SE* = .241; *Kurtosis* = 80.624, *SE* = .478] and is shown in Figure 9.

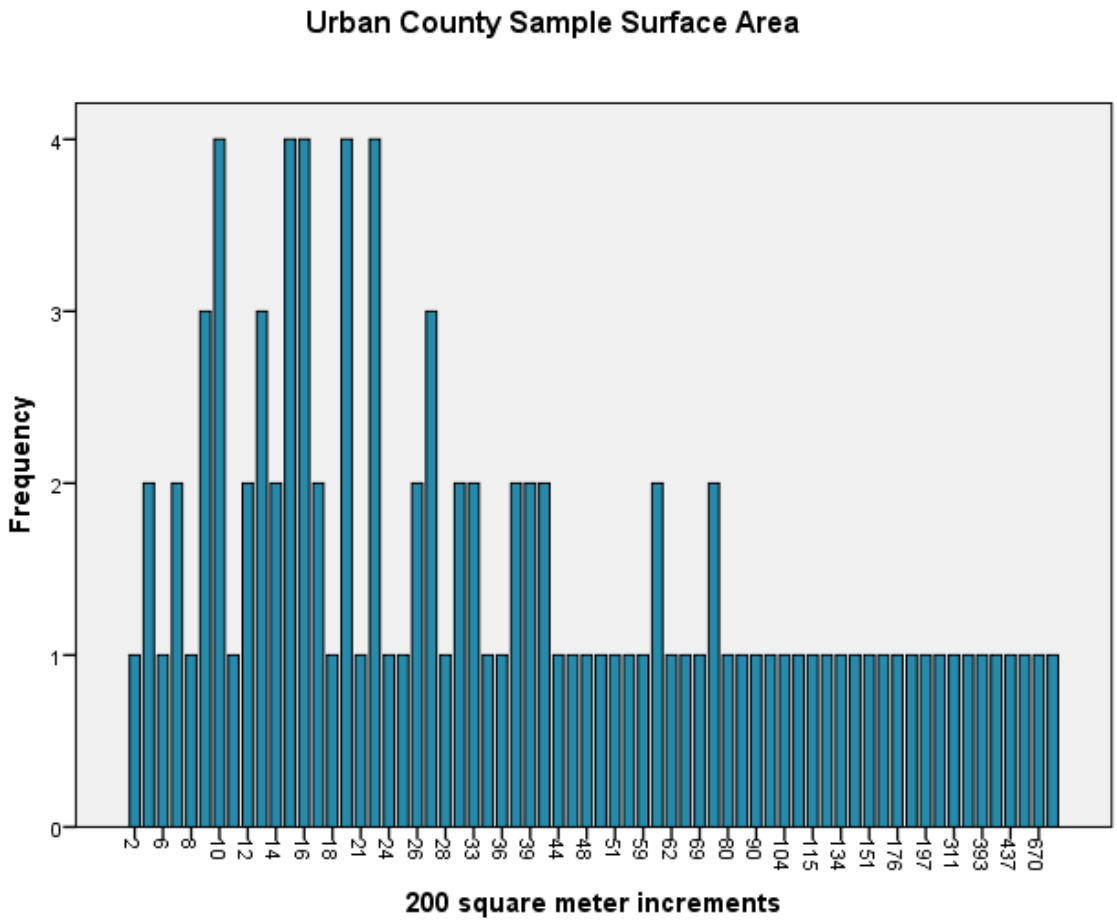

Figure 9. Frequency of urban counties' pond surface area data (surface area increment: 200 m2.).

Estimated surface area of rural ponds sampled. The rural counties' samples were collected from artificial ponds with estimated surface areas ranging from 202 m^2 to 293751 m^2 [*M* = 24319, *SD* = 52319, *Mdn* = 5733; *Skewness* = 3.525, *SE* = .266; *Kurtosis* = 13.576, *SE* = .526]. For the statistical analysis, the raw rural counties' sample surface area data was transformed into 200 m^2 increments. The transformed urban counties' sample surface areas range from 1 to 1469, [*M* = 122, *SD* = 262; *Mdn* = 20; *Skewness* = 3.525, *SE* = .266; *Kurtosis* = 13.572, *SE* = .526] and is shown in Figure 10.

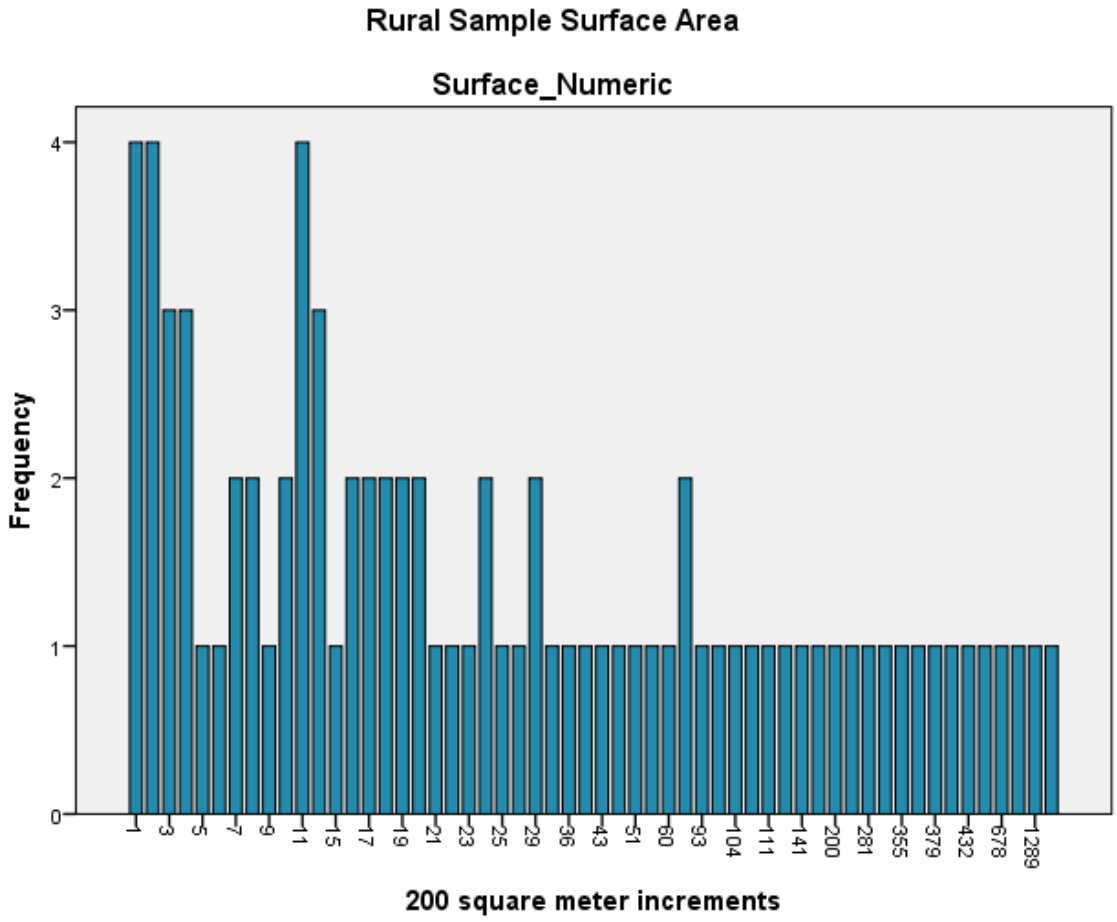

Figure 10. Frequency of rural counties' pond surface area data (surface area increment: 200 m2.).

Geochemical Properties of Ponds Sampled

The geochemical properties of the overall statewide samples were measured using an Oakton Multi-Parameter Tester 35 Series Model 35425-10 (Eutech Instruments, Thermo Fisher Scientific, Huntington Beach, California) for pH, salinity, and temperature. The overall statewide samples were collected from artificial ponds with pH measurements ranging from 7.28 to 11.19 [*M* = 9.31, *SD* = 0.744; *Mdn* = 9.29; *Mode* = 9.45; *Skewness* = -0.199, *SE* = 0.180; *Kurtosis* = -0.136, *SE* = 0.358]. The salinity

measurements of the overall statewide samples range from 23 ppm to 3270 ppm [*M* = 316, *SD* = 406; *Skewness* = 4.396, *SE* = 0.180; *Kurtosis* = 25.798, *SE* = 0.358]. The temperature measurements of the artificial ponds from which the overall statewide samples were collected ranged from 18.6 °C to 34.1 °C [*M* = 26.4, *SD* = 2.97; *Skewness* = 4.396, *SE* = 0.180; *Kurtosis* = -0.384, *SE* = 0.358]. Observational data was recorded during the field research phase of the data collection.

Geochemical properties of urban ponds sampled. The urban counties' samples were collected from artificial ponds with pH measurements ranging from 7.75 to 10.89 [*M* = 9.20, SD = 0.641; *Mdn* = 9.16; *Mode* = 8.98; *Skewness* = .086, *SE* = .241; *Kurtosis* = -.096, *SE* = .478]. The salinity measurements of the urban counties' samples range from 40 ppm to 3270 ppm [*M* = 440, *SD* = 499; *Skewness* = 3.631, *SE* = .241; *Kurtosis* = 16.570, *SE* = .478]. The temperature measurements of the artificial ponds from which the urban counties' samples were collected ranged from 20.0 °C to 34.1 °C [*M* = 26.7, *SD* = 2.61; *Skewness* = -.033, *SE* = .241; *Kurtosis* = .017, *SE* = .478].

Geochemical properties of rural ponds sampled. The rural counties' samples were collected from artificial ponds with pH measurements ranging from 7.28 to 11.19 [*M* = 9.43, *SD* = 0.840; *Mdn* = 9.60; *Mode* = 9.67; *Skewness* = -0.554, *SE* = .266; *Kurtosis* = -0.040, *SE* = .526]. The salinity measurements of the rural counties' samples range from 23 ppm to 857 ppm [*M* = 164, *SD* = 150; *Skewness* = 3.151, *SE* = .266; *Kurtosis* = 11.518, *SE* = .526]. The temperature measurements of the artificial ponds from which the rural counties' samples were collected ranged from 18.6 °C to 33.2 °C [*M* = 26.0, *SD* = 3.33; *Skewness* = 0.155, *SE* = .266; *Kurtosis* = -0.699, *SE* = .526].

Observational Data of Ponds Sampled

The observational data recorded at time of data collection included the presence or absence of waterfowl at time of sample collection and the artificial water pond setting. The frequency of the *presence* waterfowl at the time of pond water collection for the overall statewide samples was [$N_{Present}$ = 135/182 (74.2%)]. Frequency of the *absence* waterfowl at the time of pond water collection for the overall statewide samples was [$N_{absence}$ = 47/182 (25.8%)]. Artificial pond water settings were grouped into five separate categories: Business, Farm, Golf, Park, and University. As shown in Table 5 the frequencies of the pond setting for statewide samples collected were [Business = 17/182 (9.3%), Farm = 25/182 (13.7%), Golf = 71/182 (39.0%), Park = 67/182 (36.8%), and University = 2/182 (1.1%)]. The data for the descriptive statistical analyses of the pond water samples collected in urban and rural communities was selected from the dissertation study dataset specific to those counties.

Table 6

Frequency of Statewide Sample Pond Setting

Pond setting	Frequency	Percent	Valid Percent	Cumulative Percent
Business	17	9.3	9.3	9.3
Farm	25	13.7	13.7	23.1
Golf	71	39.0	39.0	62.1
Park	67	36.8	36.8	98.9
University	2	1.1	1.1	100.0
Total	182	100.0	100.0	

Observational data of urban ponds sampled. The frequency of the *presence* waterfowl at the time of pond water collection for the urban counties' samples was [$N_{Present}$ = 85/100 (85.0%)]. The frequency of the *absence* waterfowl at the time of pond water collection for the urban counties' samples was [$N_{absence}$ = 15/100 (15.0%)]. The artificial pond water settings were grouped into five separate categories: Business, Farm, Golf, Park, and University. The frequencies of the pond setting for urban counties' samples collected were [Business = 8/100 (8.0%), Farm = 7/100 (7.0%), Golf = 35/100 (35.0%), Park = 48/100 (48.0%), and University = 2/100 (2.0%)].

Observational data of rural ponds sampled. The frequency of the *presence* waterfowl at the time of pond water collection for the rural counties' samples was [$N_{Present}$ = 50/82 (61.0%)]. The frequency of the *absence* waterfowl at the time of pond water collection for the rural counties' samples was [$N_{absence}$ = 32/82 (39.0%)]. The artificial pond water settings were grouped into five separate categories: Business, Farm, Golf, Park, and University. The frequencies of the pond setting for the rural counties' samples collected were [Business = 9/82 (11.0%), Farm = 18/82 (22.0%), Golf = 36/82 (43.9%), Park = 19/82 (23.2%), and University = 0/82 (0.0%)].

Research Question (R1) Analysis

In this study the proportion and probability of the presence of influenza A virus was investigated in recirculating artificial ponds in rural and urban geographical locations. Rural ponds were viewed as one population and urban ponds as another population. The dependent *agent* variables are influenza A viruses. Influenza A virus data included: detection for M gene by real time RT-PCR using World Health Organization

recommended primer sequences, infectivity by hemagglutination assay using MDCK cell line, and nucleotide sequencing for H5N1 influenza A virus using World Health Organization recommended primer sequences at Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California).

Proportion of (+) positivity for IAV M gene sequence. The proportion of the urban counties' samples [$N_{Urban} = 100$] called (+) positive for the influenza A virus (IAV) M gene sequence [$n_u = 36$] is $P_u = 0.36$, 95% CI [0.266, 0.454]. This value is numerically larger than the proportion of the rural counties' samples [$N_{Rural} = 82$] called (+) positive for the influenza A virus M gene sequence [$n_r = 9$] is $P_r = 0.11$, 95% CI [0.042, 0.177]. It is commonly accepted the two-sided 100 (1 - α) % CI for the difference between two proportions of unequal sample size is of the form: $(\hat{p}_1 - \hat{p}_2) \pm z_{1-\alpha/2} \sqrt{[\,(\hat{p}_1 (1 - \hat{p}_1) / n_1) + (\hat{p}_2 (1 - \hat{p}_2) / n_2)\,]}$. For this analysis; [$\hat{p}_1 = n_u / N_{Urban}$], [$\hat{p}_2 = n_r / N_{Rural}$], and [$z_{1-\alpha/2} = 1.96$]. The calculated difference in the proportion of urban counties' samples called (+) positive for the influenza A virus M gene sequence to rural counties' samples called (+) positive for the influenza A virus M gene sequence is directionally towards urban counties' samples [$(\hat{p}_1 - \hat{p}_2 = 0.2502$, 95% CI (0.1278, 0.3604), $RD = 0.2502$, 95% CI (0.1337, 0.3668), $RR = 3.28$, 95% CI (1.83, 7.79), $OR = 4.563$, 95% CI (2.042, 10.193)].

The effect size index of the difference between two proportions is Cohen's h. Cohen's h is the calculated difference between the arcsine transformations of the proportions (P) of the populations (Cohen, 1988). Thus, Cohen's $h = |\varphi_1 - \varphi_2|$ where $\varphi = 2 \arcsin \sqrt{(P)}$. The calculated effect size index for the difference in the proportion of urban counties' samples to rural counties' samples called (+) positive for the influenza A

virus M gene sequence is Cohen's h = | (2 arcsine $\sqrt{(P_u)}$) – (2 arcsine $\sqrt{(P_r)}$) | = | (2 arcsine $\sqrt{(0.36)}$) – (2 arcsine $\sqrt{(0.11)}$) | = | 1.287 – 0.676 | = 0.611; a large effect size (Cohen, 1988). Using G*Power 3.1.5, the post hoc computed Power = 0.979 (two tailed) and actual $\alpha = 0.038$.

Proportion of IAV infectivity by MDCK. As aforementioned, influenza A virus (IAV) water sample infectivity was measured by hemagglutination assay using MDCK cell line. For the statistical analysis, the results of the hemagglutination titration levels-for all samples (+) positive for the influenza A virus M gene sequence-were transformed to integer values using the binary logarithm [$\log_2$ (n)] (i.e. titration level 1:16 = $\log_2$ (16) = 4). To account for negative hemagglutination (integer transformation = 0), the value of 1 is added to each integer value. Thus, the mathematical transformation is of the form: $\log_2$ (n) + 1 (i.e. titration level 1:16 = $\log_2$ (16) + 1 = 5).

An independent-samples *t* test was conducted to evaluate the burden of influenza A virus in rural ponds compared to urban ponds. The test was significant, t (26.545) = 2.330, p = .028, and favors a greater burden of influenza A virus in urban ponds over rural ponds. As shown in Figure 11, the hemagglutination titration levels transformed to integer values for urban counties' pond water samples (M = 3.08, SD = 3.210) on average were higher than for rural counties' pond water samples (M = 1.33, SD = 1.581). The 95% confidence interval for the difference in means was moderately wide, ranging from 0.208 to 3.292, which approximately represents 25% of the total range of values from 0 to 12. The effect size index for the independent samples *t* test is Cohen's d (Cohen, 1988). Cohen's d = (*Mean Difference*) / (SD_{pooled}), where $SD_{pooled} = \sqrt{[((N_1 - 1)\ SD_1^2 +}$

$(N_2 - 1)\ SD_2^2) / (N_1 + N_2 - 2)$. For this analysis, [$N_1 = n_u = 36$, $N_2 = n_r = 9$, $SD_1 = 3.210$, $SD_2 = 1.581$], thus, Cohen's d = 0.59; a medium effect size (Cohen, 1988). The eta square index (η^2) for an independent sample t test is commonly known in the form: $\eta^2 = (t^2) / (t^2 + (N_1 + N_2 - 2))$. For this analysis [$N_1 = n_u = 36$, $N_2 = n_r = 9$, $t = 2.330$], thus, the eta square index (η^2) = 0.112. The eta square index indicates that 11.2% of the variance of the hemagglutination titration levels transformed to integer values was accounted for by whether the pond water sample was collected in an urban county or a rural county. Using G*Power 3.1.5, the post hoc computed Power = 0.340 (two tailed) and Critical $t = 2.017$.

Proportion of nucleotide similarity. All of the resulting nucleotide sequences from Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California) were analyzed for genetic similarity to known influenza A viruses using the NCBI BLAST. None of the samples (N = 45) showed significant similarity to any influenza A virus strains within the databases. As shown in Table 8, all samples (N = 45) resulted "No significant similarity found". Therefore no statistical analysis comparison of the findings was conducted. Nucleotide sequencing of the samples is further detailed in section *Research question 2 analysis* of this chapter.

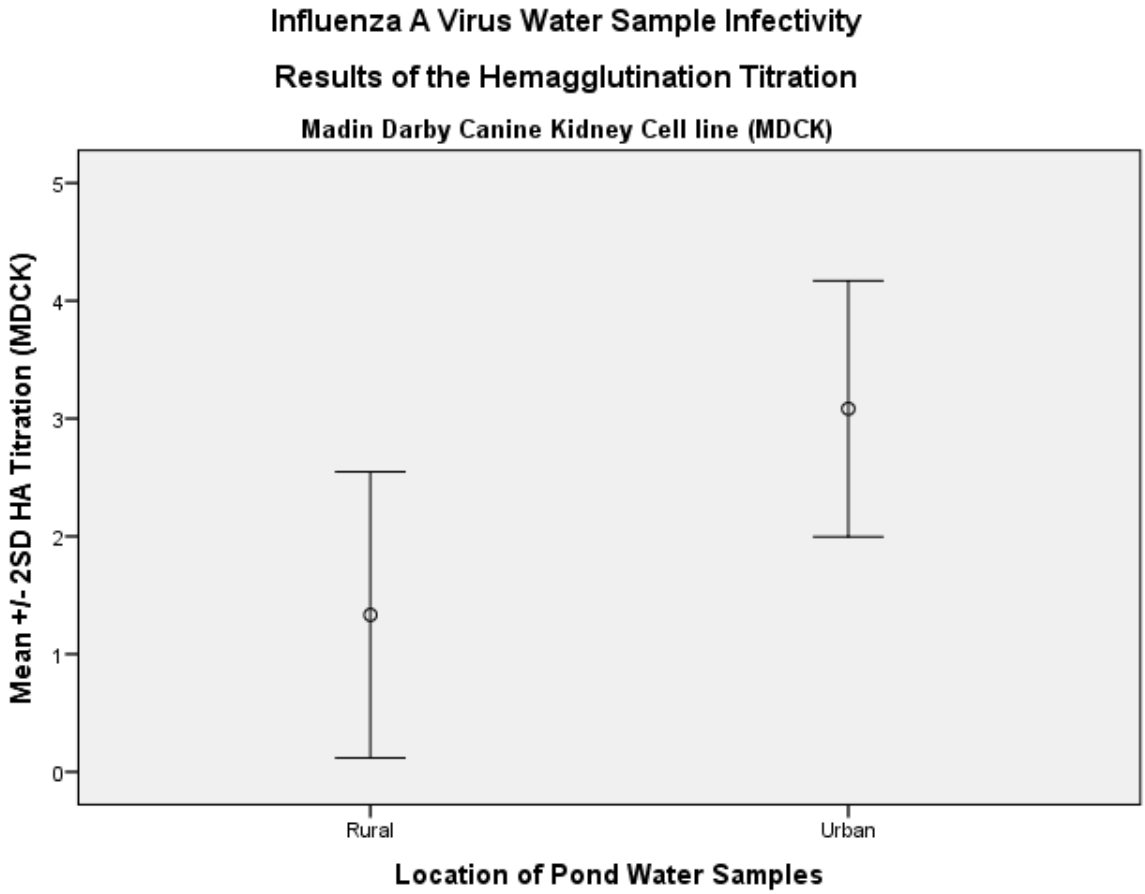

Figure 11. Transformed Hemagglutination titration values. Error bars (two standard deviations above and below the mean) for the transformed HA titration values (log2 (n) +1).

Research Question (R2) Analysis

In this study, rural community ponds were viewed as one population and urban community ponds as another population. The comparison of these two populations included proportion of influenza A virus(s), latitude and longitudinal coordinates, altitude, and approximate water pond surface area, and the presence of absence of waterfowl (*Anseriformes*) and shorebirds (*Charadriiformes*) at the time of sample collection were included as an independent *environment and host* variables. The

dependent *agent* variables were influenza A viruses. Influenza A virus data included: detection for M gene by real time RT-PCR using World Health Organization recommended primer sequences, infectivity by hemagglutination assay using MDCK cell line, and nucleotide sequencing for H5N1 influenza A virus using World Health Organization recommended primer sequences at Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California).

Association of IAV to geographic locations. A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples. The two variables were urban counties' water samples and rural counties' water samples with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. Community location and influenza A virus M gene sequence positivity were found to be significantly related, Pearson χ^2 (1, N = 182) = 15.159, p <.001, Phi = .289, p < .001. The calculated odds ratio favored urban counties' water samples [OR = 9.889, 95% CI (4.426, 22.093)]. Urban counties' water samples were nearly ten-fold more likely to be called (+) positive for the influenza A virus M gene sequence to rural counties' water samples. A similar analysis was conducted for pond water samples collected from park locations.

Association of IAV to park locations. A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples collected in park locations were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in park locations. The two

variables were urban counties' water samples collected in park locations and rural counties' water samples collected in park locations with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. Park location and influenza A virus M gene sequence positivity were found not to be significantly related, Pearson χ^2 (1, N = 67) = 0.980, p = .322, Phi = .121, p = .322. The calculated odds ratio favored urban counties' water samples collected in park locations [OR = 5.750, 95% CI (1.638, 20.183)]. Urban counties' water samples collected in park locations were nearly six-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in park locations. A similar analysis was conducted for pond water samples collected from business locations.

Association of IAV to business locations. A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples collected in business locations were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in business locations. The two variables were urban counties' water samples collected in business locations and rural counties' water samples collected in business locations with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. Business location and influenza A virus M gene sequence positivity were found not to be significantly related, Pearson χ^2 (1, N = 17) = 0.476, p = .490, Phi = .167, p = .490. The calculated 95% CI of the odds ratio implied there is no difference in water

samples collected in business locations [*OR* = 5.176, 95% CI (0.617, 43.289)]. A similar analysis was conducted for pond water samples collected from golf courses.

Association of IAV to golf course locations. A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples collected from golf courses were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected from golf courses. The two variables were urban counties' water samples collected from golf courses and rural counties' water samples collected from golf courses with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. Golf courses and influenza A virus M gene sequence positivity were found to be significantly related, Pearson χ^2 (1, N = 71) = 7.173, p = .007, Phi = .318, p = .007. The calculated odds ratio favored urban counties' water samples collected from golf courses [*OR* = 12.917, 95% CI (3.274, 50.965)]. Urban counties' water samples collected from golf courses were nearly thirteen-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected from golf courses. A similar analysis was conducted for pond water samples collected from farms.

A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples collected in farms were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in farms. The two variables were urban counties' water samples collected in farms locations and rural counties' water samples collected in farms with two levels [(+)

positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. Farms and influenza A virus M gene sequence positivity were found to be significantly related, Pearson χ^2 (1, N = 25) = 12.245, p < .001, Phi = .700, p < .001. The calculated odds ratio could not be achieved due to zero samples called (+) positive for the influenza A virus M gene sequence in rural counties' water samples collected in farms. A two-way contingency table analysis was not conducted for pond water samples collected from Universities since only two samples were collected from a University setting. Both samples were collected from a University located within a county deemed urban.

Association of IAV to presence of waterfowl

A two-way contingency table analysis was conducted to evaluate whether the presence of waterfowl at the time of overall statewide water sample collections were more likely to be called (+) positive for the influenza A virus M gene sequence to absence of waterfowl at the time of overall statewide water sample collections. The two variables were the presence or absence of waterfowl at the time of overall statewide water samples collections with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. The presence of waterfowl and influenza A virus M gene sequence positivity were found not to be significantly related, Pearson χ^2 (1, N = 182) = 2.021, p = .155, Phi = .105, p = .155. The calculated odds ratio favored statewide water samples collected in the presence of waterfowl [OR = 7.524, 95% CI (3.217, 17.595)]. Statewide water samples collected in the presence of waterfowl were more than seven-fold more likely to be called (+) positive

for the influenza A virus M gene sequence compared to statewide water samples collected in the absence of waterfowl. A similar analysis was conducted for pond water samples collected in urban county settings.

Waterfowl in urban locations. A two-way contingency table analysis was conducted to evaluate whether the presence of waterfowl at the time of urban counties' water sample collections were more likely to be called (+) positive for the influenza A virus M gene sequence compared to absence of waterfowl at the time of urban counties' water sample collections. The two variables were the presence or absence of waterfowl at the time of urban counties' water samples collections with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. The presence of waterfowl and influenza A virus M gene sequence positivity were found not to be significantly related, Pearson χ^2 (1, N = 100) = 0.054, p = .815, Phi = .023, p = .815. The calculated odds ratio favored urban counties' water samples collected in the presence of waterfowl [OR = 3.742, 95% CI (1.172, 11.947)]. Urban counties' water samples collected in the presence of waterfowl were nearly four-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to urban counties' water samples collected in the absence of waterfowl. A similar analysis was conducted for pond water samples collected in rural counties.

Waterfowl in rural locations. A two-way contingency table analysis was conducted to evaluate whether the presence of waterfowl at the time of rural counties' water sample collections were more likely to be called (+) positive for the influenza A virus M gene sequence compared to absence of waterfowl at the time of rural counties'

water sample collections. The two variables were the presence or absence of waterfowl at the time of rural counties' water samples collections with two levels [(+) positive for the influenza A virus M gene sequence, and (-) negative for the influenza A virus M gene sequence]. The presence of waterfowl and influenza A virus M gene sequence positivity were found not to be significantly related, Pearson χ^2 (1, N = 82) = 0.138, p = .711, Phi = .041, p = .711. The calculated odds ratio favored rural counties' water samples collected in the presence of waterfowl [OR = 17.000, 95% CI (3.936, 73.426)]. Rural counties' water samples collected in the presence of waterfowl were nearly four-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in the absence of waterfowl.

Association of IAV to latitude and longitude

A logistic regression analysis to investigate geographic location (latitude & longitude), altitude, and estimate surface area for statewide water sample collections was conducted. The four predictor variables were tested a priori to verify there was no violation of the assumption of the linearity of the logit. Only one of the four predictor variables, latitude, in the logistic regression analysis was found to contribute to the model B_0 = -1.113, SE_0 = .172, $Wald_0$ = 41.986, p < .001. After four iterations, the predictor variable, latitude, was included in the equation: B_1 = -.339, SE_1 = .080, $Wald_1$ = 18.142, p < .001. The estimated odds ratio favored an inverse relationship of nearly one and one-half fold [*Exp (B)* = 0.713, 95% CI (0.610, 0.833)] for every unit increase of latitude (decimal transformation). In this model, 10% to 16% [Cox & Snell R^2 = .105, Nagelkerke R^2 = .156] of the variability in (+) positive finding for the influenza A virus M gene

sequence is explained by the latitude of the water pond. The model also shows to fit the data well [Hosmer and Lemeshow's $\chi 2$ (8, N = 182) = 3.518, p = .898]. A similar analysis was conducted for pond water samples collected in urban county settings.

Latitude and longitude of urban ponds. A logistic regression analysis to investigate geographic location (latitude & longitude), altitude, and estimate surface area for urban counties' water sample collections was conducted. The four predictor variables were tested a priori to verify there was no violation of the assumption of the linearity of the logit. Only one of the four predictor variables, latitude, in the logistic regression analysis was found to contribute to the model B_0 = -.575, SE_0 = .208, $Wald_0$ = 7.627, p = .006. After three iterations, the predictor variable, latitude, was included in the equation: B_1 = -.295, SE_1 = .113, $Wald_1$ = 6.767, p = .009. The estimated odds ratio favored an inverse relationship of nearly one and one-half fold [*Exp (B)* = 0.745, 95% CI (0.597, 0.930)] for every unit increase of latitude (decimal transformation). In this model, 7% to 10% [Cox & Snell R^2 = .069, Nagelkerke R^2 = .094] of the variability in (+) positive finding for the influenza A virus M gene sequence is explained by the latitude of the water pond. The model also shows to fit the data well [Hosmer and Lemeshow's $\chi 2$ (8, N = 100) = 5.831, p = .666]. A similar analysis was conducted for pond water samples collected in rural county settings.

Latitude and longitude of rural ponds. A logistic regression analysis to investigate geographic location (latitude & longitude), altitude, and estimated surface area for rural counties' water sample collections was conducted. The four predictor variables were tested a priori to verify there was no violation of the assumption of the

linearity of the logit. After five iterations, none of the four predictor variables; latitude, longitude, altitude, and estimated surface area, in the logistic regression analysis was found to contribute to the model B_0 = -2.093, SE_0 = .353, $Wald_0$ = 35.106, $p < .001$ and thus, no equation was calculated.

Research question (R3) analysis

(R3)- Is there an association between water pH, salinity, or water temperature and influenza A virus infectivity in rural and urban water ponds?

A multivariate linear regression analysis was conducted to investigate the hypothesis of an association between pond water sample pH, salinity, and water temperature at time of collection and influenza A virus infectivity. Samples for this analysis-selected from the overall statewide sample data set-were verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence. The number of statewide samples [N = 182] verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence is [n = 45]. The predictors of this analysis are: pond water pH, pond water salinity, and pond water temperature at time of collection. The pH of the pond water samples-at time of collection-range from 7.89 to 10.52 with [*M* = 9.24, *SD* = 0.66]. The salinity of the pond water samples-at time of collection-range from 23 ppm to 3270 ppm with [*M* = 465, *SD* = 646]. The water temperature of the pond water samples-at time of collection-range from 18.6°C to 31.0°C [*M* = 26.3, *SD* = 2.68]. The dependent variable of this analysis is influenza A virus infectivity. As noted above, the results of the overall statewide samples verified for their content of influenza A virus genome by real time RT-PCR targeting the

M gene sequence are [Neg = 17/45 (37.8%), 1:1 = 6/45 (13.3%), 1:2 = 2/45 (4.4%), 1:4 = 5/45 (11.1%), 1:8 = 1/45 (2.2%), 1:16 = 4/45 (8.9%), 1:32 = 4/45 (8.9%), 1:64 = 1/45 (2.2%), 1:128 = 2/45 (4.4%), 1:256 = 3/45 (6.7%), 1:512 = 0/45 (0.0%), 1:1024 = 0/45 (0.0%), 1:2048 = 0/45 (0.0%)].

As previously aforementioned in Chapter 3, influenza A virus water sample infectivity was measured by hemagglutination assay using MDCK cell line. Serial dilutions of the samples were assayed for hemagglutination of MDCK ranged from 1:1 to 1:2048. The highest hemagglutination titration was recorded per sample. If no hemagglutination of MDCK was microscopically identified, the sample was deemed "Negative". For the statistical analysis, the results of the hemagglutination titration levels-for all samples verified (+) positive for the influenza A virus M gene sequence-were transformed to integer values using the binary logarithm [$\log_2(n)$] (i.e. titration level 1:16 = $\log_2(16) = 4$). To account for negative titration levels (integer transformation = 0), the value of 1 is added to each integer value. Thus, the mathematical transformation is of the form: $\log_2(n) + 1$ (i.e. titration level 1:16 = $\log_2(16) + 1 = 5$).

Statistical assumptions

The predictors for this multivariate linear analysis were tested for the assumption of linearity. As shown in Figure 12, there appears to be a linear increase in hemagglutination titer with increase in pond water sample pH. As shown in Figure 13 and 14 the hemagglutination titer did not increase or decrease with increase pond water salinity or pond water temperature, respectively. The horizontal lines produced are linear, but horizontal to the X-axis. The cross-sectional random sampling strategy of the

statewide pond water samples satisfies the assumption of sampling independence. As shown in Figure 15, the dependent variable hemagglutination titer appears not to be normally distributed.

Figure 12. Scatterplot diagram of Pond Water pH to Hemagglutination titer

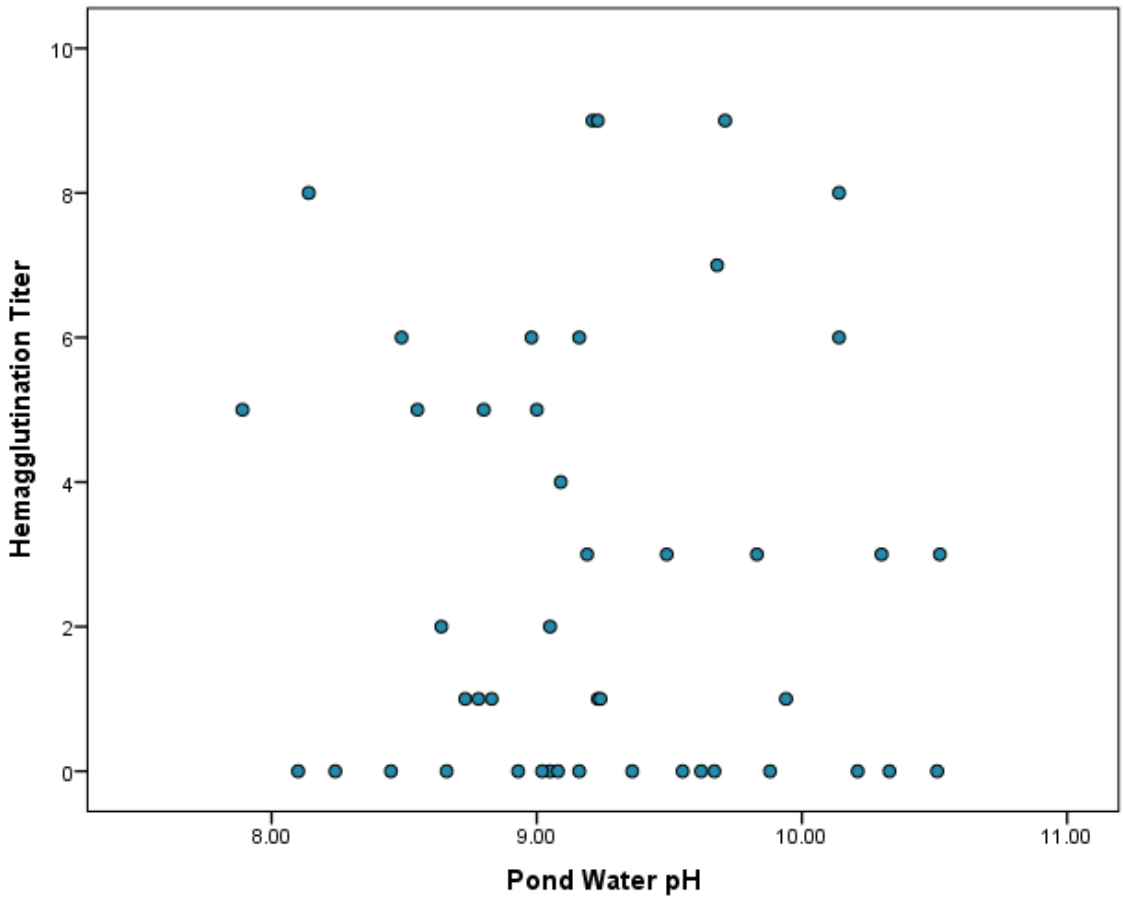

Figure 12. There appears to be a linear increase in hemagglutination titer with increase in pond water sample pH.

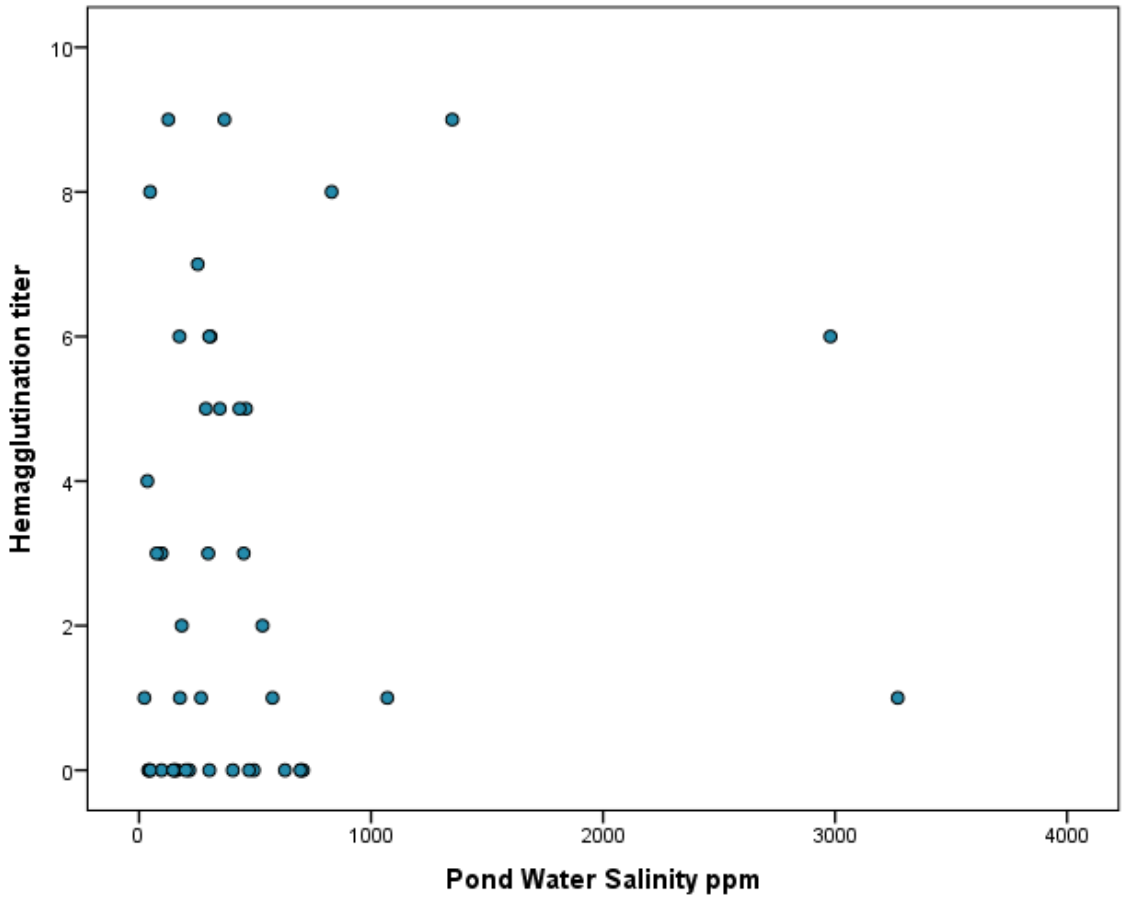

Figure 13. Scatterplot diagram of Pond Water Salinity to Hemagglutination titer. The hemagglutination titer does not increase or decrease with increase pond water salinity (ppm).

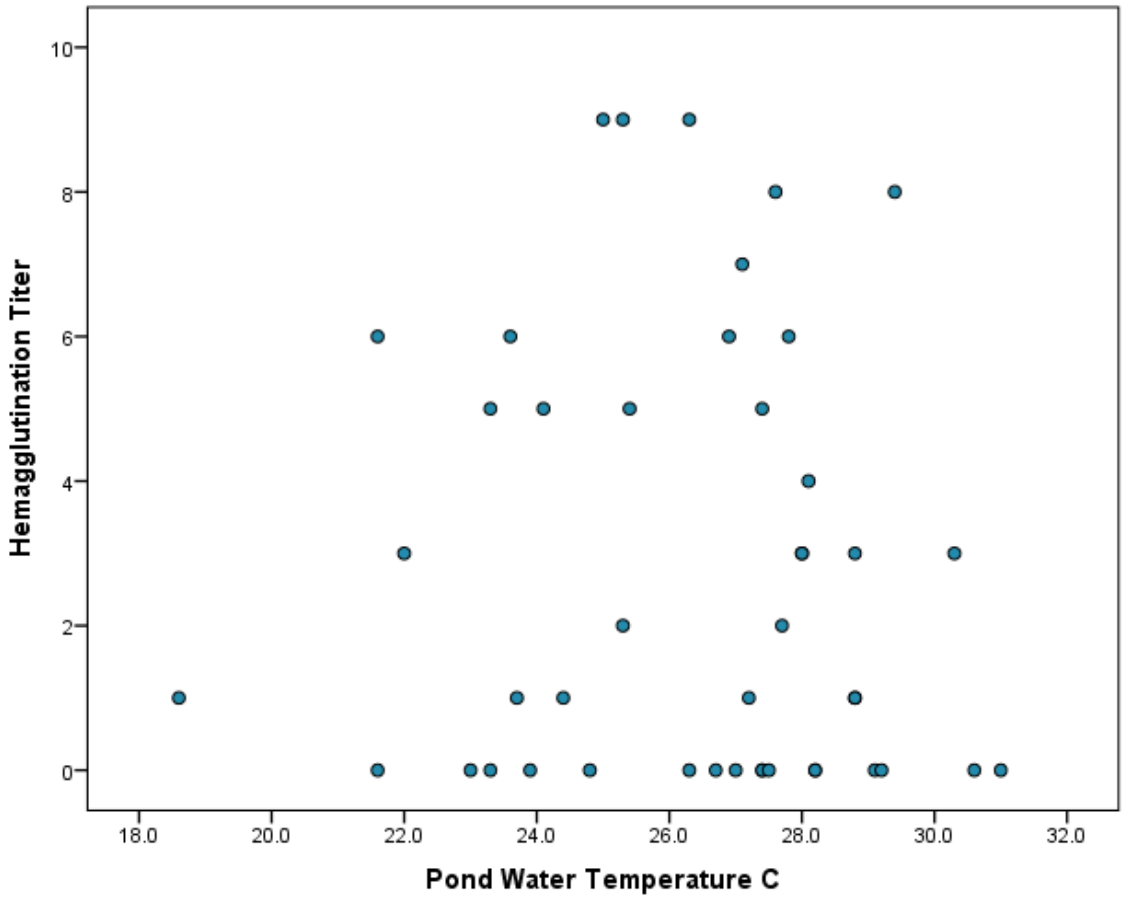

Figure 14. Scatterplot diagram of Pond Water Temperature to Hemagglutination titer. The hemagglutination titer does not increase or decrease with increase pond water temperature

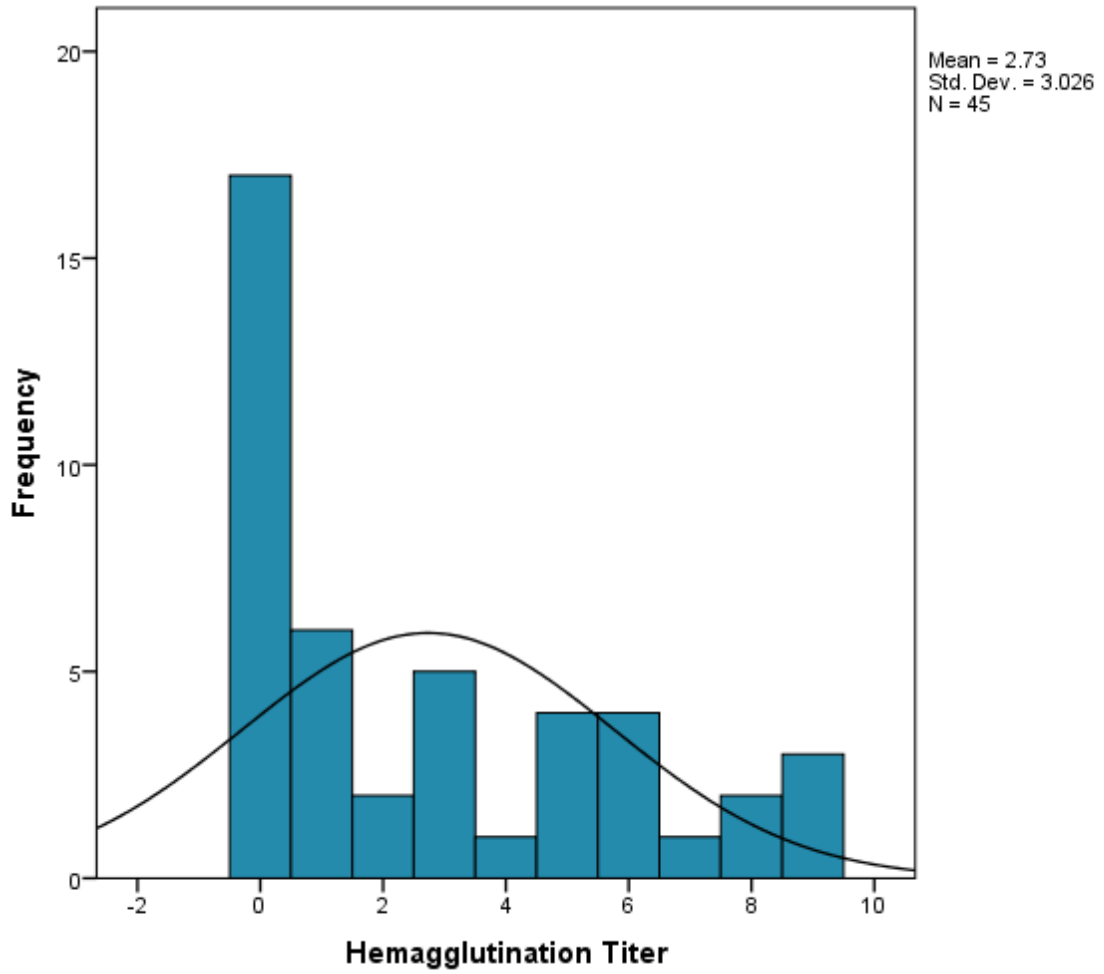

Figure 15. Histogram of Hemagglutination Titer Madin Darby Canine Kidney cell line. The dependent variable hemagglutination titer (MDCK) appears not to be normally distributed.

Statistical analysis. In the model summary for this analysis, the R is slightly positive (R = 0.112), so there is a slight positive correlation between pond water sample pH, salinity, or water temperature, and influenza A virus infectivity. Since the value of R is close to zero, the correlation between all the variables and influenza A virus infectivity is not strong. The coefficient of determination is [R^2 = .013]. Based on this, only 1% of the variability in hemagglutination titer is explained by pond water sample pH, salinity,

or water temperature at time of collection. Hence, 99% of the variability maybe explained other variables not investigated in this study.

In the coefficients table, none of the three independent variables was found to be significant predictors of change. Controlling for pond water pH and salinity, for each 1.0°C increase in temperature, the binary logarithm [$\log_2(n)$] value for hemagglutination titer decreased by -.064. Controlling for pond water pH and temperature, for each unit (ppm) increase in salinity, the binary logarithm [$\log_2(n)$] value for hemagglutination titer remained unchanged. Controlling for pond water salinity and temperature, for each 1.0 increase in pH, the binary logarithm [$\log_2(n)$] value for hemagglutination titer increased by 0.019. None of the three predictor effects were statistically significant and all had confidence intervals that included 0: pH [ß = 0.019, 95% CI (-0.440, 0.312), p = .980], salinity [ß = 0.000, 95% CI (-0.001, 0.002), p = .570], temperature [ß = -0.064, 95% CI (-0.440, 0.312), p = .733]. As previously noted, due to the unexpected delays in preparation and testing for hemagglutination using MDCK cells in combination with a lack of a positive influenza A virus control the results of the hemagglutination MDCK plaque assay should be interpreted with caution.

Research Question (R4) Analysis

The samples [N = 45] verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence were submitted to Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California). All [N = 45] samples verified for their content of influenza A virus genome by real time RT-PCR targeting the M gene sequence were also (+) positive for H5 or N1 or both. To submit

samples, 15 μml of the amplification product of the real time RT-PCR targeting the H5 gene sequence was extracted and transferred to a clean, labeled 1.5 ml sample centrifuge tube.

As previously noted, all of the resulting nucleotide sequences from Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California) were analyzed for genetic similarity to known influenza A viruses [GenBank (National Institute of Health genetic sequence database)] using the NCBI BLAST. A universal forward primer set for sequencing HA_0 developed by Gall, Hoffmann, Harder, Grund, and Beer (2008) and a forward primer sequence for N1 identified by Huang, Khan, & Măndoiu (2013) were utilized to sequence the influenza A virus samples[N = 45] for this dissertation study. The primer sequences were manufactured by Invitogen (Carlsbad, California) and are shown in Table 6 and Table 7. All samples (N = 45) were searched using the limited records matching entrez query: txid197911 [ORGN] OR txid11320 [ORGN] OR txid11320 [ORGN] OR txid102797 [ORGN] OR txid102797 [ORGN]. None of the samples (N = 45) showed significant similarity to any influenza A virus strains within the databases. As shown in Table 8, all samples (N = 45) resulted “No significant similarity found”.

Table 7

Universal Forward Primer Set for Sequencing HA_0

Oligonucleotide	Sequence (5′-3′)*a*	Position (nt)
HA-1057.1-F	GGR GAA TGC CCC AAA TAY GT	967-986
HA-1057.2-F	GGR ARA TGC CCC AGR TAT GT	967-986
HA-1057.3-F	GGR GAA TGC CCC AAR TAY AT	967-986

Table 8

Forward Primer for Sequencing N1

NA Subtype	Sequence (5′-3′)*a*	Product length (bp)
N1 - Forward	5'-TAGACTGCATGAGGCCTTGCTTCTG-3'	137

Table 9

HA and NA Gene Sequencing Results with BLAST Analysis Results

Sample ID.	HA gene sequencing NA gene sequencing	
C-1001	NNNNNNNNNNNNNNNNNNNNNNGNNNNNNNN NNNNNNNNNNNNNGNTNNGNGNGNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1003	NNNNNNNNNNNNNNNNNNNGNNNNNNNGNN NNGGGNNNNNNNNNNANNNNNN NNNNNNNNNNNNNGNNNGGNNGNNNNNNNNN	No significant similarity found
	NNNNGGGNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNN	No significant similarity found
C-1006	NNNNNNNNNNNNNNNNNNNNNNNGNNNNNNN NNNNNNNNNNNNNNNNNNCCNNCCNGNNNNC NNCNCNNNNNNNCNNNCNCNNNNNNCNNCCNC CCNCCCNCCCCCCNATGNNNNNCCCNCCNNNC CCTCNCCNNNCNATCCCCCCCCCNNAANN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNGNNGNNTNNN NNCNNNATACNCNNNNCNGNNNNGN	No significant similarity found
C-1007	NNNNNNNNNNNNNNNNNNNNNNGGGGGGNN GGNNNNNNNNNNNCNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNGNNNTNNNNACNCTNTCANNAGTTGGNG	No significant similarity found
C-1009	NNNNNNNNNNNNNNNNGNGNGGGGNNNNNNN NNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNTNNNGNNNNNN NNNNNNNNNNNGNGNTGNNNNNNNNNNNNNN NNTNTTTTNTTNNNNNNANTNNTCTNNN	No significant similarity found
C-1010	NNNNNNNNNNNNNNNNNNNNNNGNGNANNGN NNNNNNNNNNNNNNNNNNAANAGGNN	No significant similarity found
	ANNNNNNNNNNNNNNNNNNGNNNCNNTCNNA GTNGNAGNTATANATA	No significant similarity found
C-1011	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNGNNNNNNNNNNNNNNNNANNN	No significant similarity found
	NNNNNNNNNNNNNNNNTNNGNAGNNNNNNNNN TTNNATNNNNNNNNNNNNG	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1012	NNNNNNNNNNNNNNNNNNNGGGGGGGNNNN GNNNNNNNNNNNNNNCTCCNNNNTNNNNNGNN NNNNCCNNNCCCNCNNNNAATTNTTNTTTTTNN NNNNNNNNNNNNNNNNNNNCNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1013	NNNNNNNNNNNNNNNNNNNNNNNGNNNNGN NNNNNNNNNNNNNNNNNNNNNNNNNNCCTA GNCNNNTNTTTTNTNNNATGNNNTNCNCCNNNN NNNNNNNANN	No significant similarity found
	NNNNN	No significant similarity found
C-1015	NNNNNNNNNNNNNNNGNGNNNNGGGGNN NNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNGNNNNGNNNNNNNNNNN NGGGNNNNNNNGNNNNNNNNNNNNNNNNT NNNNANTNNNNNNNNTNTCAGATTGGA	No significant similarity found
C-1017	NNNNNNNNNNNNNNNNNNGGGGGGNNNGGNN NNNNNNNNNNNNNNNNNNNNNNNG	No significant similarity found
	NNNNNNNNNNNNNNNGGNNGNNGNNNNNNNN GGGGGNGNNNNNNNNNNNNNNNNNNTNNNNN NNNNNNNNNANNNTCAGNTTGGAGGATNNNNN NN	No significant similarity found
C-1020	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNG NNNNNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNACNCNNCAGANTTGNNGNNT NNCNTA	No significant similarity found
C-1021	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NTNNNTNNNNNNNNNTNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNTGNGNNNN NNNNNNAANATANATANNNNN	No significant similarity found
C-1024	NNNNNNNNNNNNNNNNNNNNGNGGGGNNNGN NNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNTNN NNNNNNNNNAGNNTNNNNN	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1026	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGN NNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGNNNN NNNNNNNTTNNGNNNNNNNNNNNNNNNNNN NNNNNNNNNNANNNNNNNNNNNNNNNNNN NNNNATNNNNNNNNNNN	No significant similarity found
C-1028	NNNNNNNNNNNNNNNNNNNNNNGGNNNGNNN NNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1030	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGNN NGGGNNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1033	NNNNNNNNNNNNNNNNNNNNNGANNNNNNN NNNNNNNNNNNNNNANNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1034	NNNNNNNNNNNNNNNNNNNNNNNNNNNTTNN NNTTTNNNNNNNNNNNNNNNNNNNNNNNN NNNNCNNNNNNNTNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNTNN NNANNNNCNTGACNCNNNCNNNGGTNGNNGNN TNNCNTANN	No significant similarity found
C-1039	NNNNNNNNNNNNNNNNNNGNNNNNNNNNGNG NNNNNNNNNNNGGNNNNNNNNNNNNNNNNN GNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNGNNNNNTNNNNNN NNAANANNNNNNANGN	No significant similarity found
C-1041	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNG NNNNGGNNNNNNNNNNNNNNNNNNNNNN NNNATANNNNNNTTANNNN	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1043	NNNNTNNNNNNNNNNNNNNNNNNNNNGAN NNNNNNNNNNNNNNNNNNNNNCNAN	No significant similarity found
	NNNNN	No significant similarity found
C-1045	NNNNNNNNNNNNNNNNNNNNNNNNGNGNTAN NNNNNNNNNTNNCNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1048	NNNNNNNNNNNNNNNNNNNNNGNNNNNNNNNN NNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNGGNGNGNNGNNGNNNN GNGNNNNNGNNNGNNNNNNNNNNNNGNNNNNN NNTNNNNNNNNNNNNNNCNNGN	No significant similarity found
C-1052	NNNNNNNNNNNANNNNNNNNNNNNNNNNGN NNNNCNNNNNNNNNNNNNNNNNNNCNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NTNNNNNNNNNNATNNNNNNCNNNNNTNGAGN NTNNCNTA	No significant similarity found
C-1054	NNNNNNNNNNNNNNNNNNNNNNNGNNNGNNN NNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1057	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NGNNNNNNNNNNNNNNNCNNNCNNNNNNNNN N	No significant similarity found
	NNNNN	No significant similarity found
C-1061	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGGT TNTNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNG NNNGNNNNNNNNNNNNNNNNNNNNNNTNNN NNNNNN	No significant similarity found
C-1067	NNNNNNNNNNNNNNNNNNNNNNNNNNGNTNNN NNNNNNNNNNGNNNNNNCNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1069	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGGG NNNNNNNNNNNNNNNNNNA	No significant similarity found
	NNNNN	No significant similarity found
C-1078	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGNGN NNNNNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGN GGNNNNNCNNNNNNNNNNNNNNNNNNNNA NTN	No significant similarity found
C-1081	NNNNNNNNNNNNNNNNNNNNNNNNNGGGGNN NGNNNNNNNNNNNNNNNNNNAN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNGNNTCTGT NGNGTGTTNTANNNTNCATA	No significant similarity found
C-1085	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1088	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN TNNNNTNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNGGNNNNNGNNNNNNNN GGGGGGNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNN	No significant similarity found
C-1092	NNNNNNNNNNNNNNNNNNNGNNGNNNNNTNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1095	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NTNNNNNNNNNNNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNCNNGNNNNNNNNAG NNNNNTANNNNNNNNNNNN	No significant similarity found
C-1101	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGNGN NNNNNNNNNTNNNNNNCA	No significant similarity found
	NNNNN	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1104	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNGNNNNNANNNCNNNNNNNNNNNANNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNGNNGNNN NGGNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNTANGTNTC	No significant similarity found
C-1111	NNNNNNNNNNNNNNNNNGGGGGGGNNTNTG NNNNNNNNNNNNNNNNNNNNANGATCNGAN NNTNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNTN NNTNTNTNATTNNNTANATNN	No significant similarity found
C-1129	NNNNNNNNNNNNNNNNNNNNTNNNGNNNNNN NNNNNNNNNNTNNANNNGNNNNNNNNNNTT NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNTNNNNNNN N	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNGNNTTGGNNNNNNNNNNNNNNNNNCNTN NNTTNGGT	No significant similarity found
C-1136	NNNNNNNNNNNNNNNNNNNNNGNGGGGNNN NGNNNNNNNNNNNNNNNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN NNNNNNTGNTACACNNTCNTGNTTGNNGNNNN NTANNN	No significant similarity found
C-1149	NNNNNNNNNNNNNNNNNNNNNNNNNNNGNNN NNNNNNNNNNNNNNNNNNNNNNANNNNN	No significant similarity found
	NNNNN	No significant similarity found
C-1168	NNNNNNNNNNNNNNNNNNGNGNNGNNTNNNN NNNNNNNNNNNNNNNNNNNNNNNNNCNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNTTGNGTTNTNNNNTA TAACTCCTCCNGCG	No significant similarity found

(table continues)

Sample ID.	HA gene sequencing NA gene sequencing	
C-1169	NNNNNNNNNNNNNNNNNNNNNNGGNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNGNNN	No significant similarity found
	NNNNNNNNNNNNNNNNNNNNNNNNNNNNGNN NNNNNTANNNTATACNCTNTCANNNTNGNGGN TNCNTAN	No significant similarity found

Note. Nucleotide sequencing results from Laragen Sequencing and Genotyping Laboratory (Laragen, Inc., Culver City, California) and NCBI Blast analysis results for similarities to known influenza A virus strains. Sequence base pair codes (nitrogenous bases): A = Adenine, T = Thymine, C = Cytosine, G = Guanine, and N = Non-identified nitrogenous base.

Summary

This dissertation study has been an investigation into the persistence of influenza viruses in artificial suburban neighborhood water ponds. No known research has analyzed the proportion and persistence of influenza viruses in these aquatic habitats. The aim of this dissertation study was to investigate the proportion of influenza A viruses in recirculating artificial ponds in rural and urban geographical locations. To investigate the proportion of influenza A viruses in recirculating artificial ponds, 182 pond water samples were collected from a representative sampling from 14 counties considered rural areas ($N_{Rural} = 82$), and 25 counties considered metropolitan and not rural ($N_{Urban} = 100$) in California by California Business and Professions Code Section 19986(l) was achieved. Field research data and laboratory data were transcribed to an Microsoft Office Excel 2007 spreadsheet and statistically analyzed to answer the four research questions of this study.

The analysis of the proportion of influenza A virus in rural and urban water ponds favored the greater burden of influenza A virus in urban community ponds over rural community ponds [($\hat{p}_1 - \hat{p}_2$ = 0.2502, 95% CI (0.1278, 0.3604), Cohen's h = 0.611, Power = 0.979, actual $\alpha = 0.038$]. An independent-samples *t* test was conducted to evaluate influenza A virus water sample infectivity in samples from rural and urban water ponds. The test was significant, t (26.545) = 2.330, p = .028, Cohen's d = 0.59, eta square index (η^2) = 0.112, and also favored a greater burden of influenza A virus in urban ponds over rural ponds. Factors of pond water location and setting were also analyzed as predictors.

A two-way contingency table analysis was conducted to evaluate whether urban counties' water samples were more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples in five different settings: businesses, farms, golf courses, parks, and Universities. Overall, urban counties' water samples were nearly ten-fold more likely to be called (+) positive for the influenza A virus M gene sequence to rural counties' water samples [*OR* = 9.889, 95% CI (4.426, 22.093)]. The calculated 95% CI of the odds ratio implied there is no difference in water samples collected in business locations [*OR* = 5.176, 95% CI (0.617, 43.289)]. The calculated odds ratio for pond water samples collected in farms could not be achieved due to zero samples called (+) positive for the influenza A virus M gene sequence in rural counties' water samples collected in farms. However, farms and influenza A virus M gene sequence positivity were found to be significantly related, Pearson χ^2 (1, N = 25) = 12.245, $p < .001$, Phi = .700, $p < .001$. Urban counties' water samples collected from golf

courses were nearly thirteen-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected from golf courses [*OR* = 12.917, 95% CI (3.274, 50.965)]. Urban counties' water samples collected in park locations were nearly six-fold more likely to be called (+) positive for the influenza A virus M gene sequence compared to rural counties' water samples collected in park locations [*OR* = 5.750, 95% CI (1.638, 20.183)]. A two-way contingency table analysis was not conducted for pond water samples collected from Universities since only two samples were collected from a university setting within an urban county. The presence of waterfowl and three geochemical measures of the pond water samples were analyzed as predictors.

The presence of waterfowl and influenza A virus M gene sequence positivity were found not to be significantly related in the overall statewide samples, urban counties' samples, and the rural counties' samples. However, the calculated odds ratio favored rural water samples collected in the presence of waterfowl for all three groups: the overall statewide samples, urban counties' samples, and the rural counties' samples. Latitude was shown to be a predictor of (+) positivity for the influenza A virus M gene sequence for statewide and urban counties' pond water samples, but not for rural counties' pond water samples. Longitude, altitude, and estimated surface area were not found to contribute to any of the models. The analysis of the geochemical properties; pH, salinity, and water temperature at time of collection of the pond samples did not identify any predictors of influenza A virus infectivity. None of the samples (N = 45) submitted for nucleotide sequencing and subsequent strain identification showed significant similarity to any

influenza A virus strains within GenBank (National Institute of Health genetic sequence database). Therefore no statistical analysis comparison of the findings of the NCBI BLAST was conducted.

Chapter 5 includes a discussion of the interpretations of the research data. The discussion begins with an interpretation of the findings as related to the literature, as well as, related to ecological and molecular structure topics. This section is followed by a presentation of the limitations of this research study, and then a discussion of recommendations for further research. These sections are followed by a discussion of the potential social change impact of this dissertation study at the international level, the national and state level, the population level, and at the individual level.

Chapter 5: Discussion, Conclusions, and Recommendations

The "Spanish" influenza H1N1 pandemic of 1918–1919, which caused approximately 50 million deaths worldwide, remains an ominous warning to public health (Taubenberger & Morens, 2006). Since then, new subtypes of human influenza A viruses have been detected at various times: in 1957, the H2N2 subtype (Asian influenza) replaced the H1N1 subtype; in 1968, the H3N2 subtype (Hong Kong virus) appeared; and in 1977, the H1N1 virus reappeared (Webster et al., 1992). Influenza disease emergence data are collected year-round, but economic strain on global public health to prevent and treat human influenza outbreaks is enormous. Therefore, it is imperative to identify potential sources of the virus to help minimize outbreak occurrence.

There are gaps in knowledge about the association between molecular structure, epidemiologic and clinical characteristics, and the impact of ecological and other contextual aspects of influenza viruses. Gaps in understanding the role of the physical and biogeochemical environment as an integral part of the influenza A viral transmission also exist (Lang, Kelly, & Runstadler, 2008). More importantly, gaps in knowledge about the burden of influenza A virus in rural and urban community settings remain present. IRAT is expected to prompt additional studies to address these key gaps in the knowledge (Trock, Burke, & Cox, 2012).

The problem in this study was that public health scientists have been battling emerging human influenza diseases with tactile and reactionary methods because there was a lack of knowledge and data at the human-animal interface. This baseline study of the proportion of influenza A virus in urban and rural community settings may provide

knowledge and biological data of significant interest at the human-animal interface. This biological data may be of significant interest for the development of IRAT.

Interpretation of the Findings

The results of this dissertation study showed an association in the burden of influenza A virus to geographical location. The analysis of the data favored a greater burden of influenza A virus in urban ponds compared to rural ponds. Additionally, this dissertation study's findings aligned with the research of others who investigated the persistence of influenza A virus in aquatic habitats. The interpretation of the data analysis—MDCK plaque assays, real time RT-PCR, and nucleotide sequencing—supported the proposal by Franklin et al. (2011) that aquatic systems may serve as reservoirs and sources of infection for both wild birds and mammals. That being said, the aquatic habitats sampled for this study were potential sites of the human-animal interface of influenza A viruses. Interestingly, the analysis of the dissertation data did not fully support the well-accepted theory of the influenza virus human-animal interface by Webster et al. (1992).

As aforementioned, Webster et al. (1992) described the aquatic habitat as an influenza virus human-animal interface with the example of domestic ducks in community ponds attracting migratory waterfowl. The migratory waterfowl introduce the influenza virus to that community's water pond from fecal contamination. The contaminated community water pond now becomes a potential source of influenza virus to both humans and animals. In this dissertation study, the presence or absence of waterfowl did not show to be a statistically significant factor for the detection of

influenza A virus. However, the calculated odds ratio favored pond water samples collected in the presence of waterfowl to be called (+) positive for the influenza A virus M gene sequence compared to absence of waterfowl at the time of pond water sample collection. This discrepancy maybe explained by the work by Ito et al. (1995). The results of the Ito et al. investigation showed the influenza A viruses remained viable in the lake water after most ducks left for migration south. In this study, there was no credible evidence that the presence of waterfowl at time of collection contributed to the sample being verified as (+) positive for the influenza A virus M gene sequence. Therefore, the presence or absence of waterfowl-as tested in this dissertation study may not be a contributing factor for the detection of influenza A virus. Geospatial analysis also did not show to be a strong contributing factor for the detection of influenza A virus.

The latitude of the sampled water pond was shown to be a predictor of (+) positivity for the influenza A virus M gene sequence. In contrast, longitude, altitude, and estimated surface area were not found to contribute to any of the statistical models. Additionally, the laboratory investigation by Brown et al. (2009) indicated the pH, temperature, and salinity at levels normally encountered in nature can impact the ability of avian influenza A viruses to remain infective in water, but this could not be replicated in the field. The analysis of the geochemical properties—pH, salinity, and water temperature at time of collection of the pond water samples—did not identify any predictors of influenza A virus infectivity. This dissertation study did support some of the material and methods identified in the literature.

Evers et al. (2007) investigated the commercial preservative RNAlater (Qiagen®) against the current method of cryo-freezing, and ethanol preservatives for influenza A virus samples. The purpose of Evers et al.'s investigation was to determine if using the commercial preservative RNAlater would result in improved RT-PCR amplification over the current sample preservation methods of cryo-freezing or ethanol fixation. In this dissertation study, the commercial preservative RNAlater was successfully used as a stabilizer to protect RNA for subsequent analysis. However, sample preservation methods of cryo-freezing or ethanol fixation was not compared. Zhang et al. (2011) collected 200-ml water samples from areas near the habitat of migratory birds and stored the samples at -80°C until assayed. In this dissertation study, a 200-ml to 250-ml pond water sample was successfully collected and analyzed from each site. Tønnessen et al. (2013) collected samples along migratory flyways for influenza viruses, genome sequencing, and similarity analysis.

In this dissertation study, I successfully collected pond water samples along the eastern Pacific flyway and analyzed the samples for influenza A viruses, genome sequencing, and similarity analysis. The positive results of the HA_0 and N1 nucleotide sequencing are viewed as validation for the real time RT-PCR analysis and also as validation for the presence of influenza A virus in the pond water samples. The HA_0 and N1 nucleotide sequencing was performed on the real time RT-PCR amplification products of the samples ($N = 45$) that verified (+) positive for H5 or N1 by real time RT-PCR. A positive HA_0 and N1 nucleotide sequencing result should only occur if amplified hemagglutination or neuraminidase protein strains are present in the sample. The

amplified hemagglutination or neuraminidase protein strains should only be present in the nucleotide sequencing samples if the proteins were amplified during the real time RT-PCR assays. For the parameters of this study, the real time RT-PCR assay should only be able to amplify the (H5) hemagglutination or (N1) neuraminidase protein strains if either influenza A virus H5 RNA strains or influenza A virus N1 RNA strains were present in the collected pond water samples. Therefore, the positive results of the HA_0 and N1 nucleotide sequencing are validation for the presence of influenza A virus in the pond water samples along the eastern Pacific flyway in California. Further, these (+) positive influenza A virus pond water samples originated from a human-animal interface.

Ecological Interpretations

The study and detection of influenza A virus along the eastern Pacific flyway were in alignment with the World Health Organization (2011) description of the human-animal interface as a complex juncture at which new paradigms are emerging. Austin and Hinshaw (1984) suggested that surveillance of healthy ducks and the aquatic environment they frequent may be of significant interest to monitoring and controlling influenza A viruses. This dissertation study is a model that can be modified to monitor influenza A virus in the aquatic environment. Ferro et al. (2010) focused on the presence of influenza A viruses among the migratory ducks and the winter breeding grounds along the Texas coast. As previously mentioned, in this dissertation study pond water samples were collected along the eastern Pacific flyway and analyzed for influenza A viruses. Lang et al. (2008) collected and studied sediment samples for influenza A virus from three ponds in Alaska using a time-series approach. Hinshaw et al. (1980) completed a similar

longitudinal study to isolate influenza viruses and to determine whether influenza viruses continually circulate or whether the same or different strains are present from year to year. A time series approach would be an appropriate modification to the cross-sectional baseline approach of this dissertation study.

Many have studied the theory of water-borne transmission of influenza A viruses among waterfowl (Brown et al., 2009; Halvorson et al., 1985). The findings of this dissertation study cannot adequately address the theory of water-borne transmission of influenza A viruses among waterfowl. However, replication of this research model may provide data on the size of the gene pool of influenza A viruses in artificial aquatic habitats in California and the number of viruses antigenically related to human strains present in avian species that use these habitats. The findings of this dissertation study showed there was a greater burden of influenza A virus in aquatic habitats of urban communities. Future research of this human-animal interface could use these geographical findings to locate and limit the areas of interest.

Molecular Structure Interpretations

The results of this dissertation study add to the body of knowledge of the laboratory analysis of influenza viruses. As previously discussed, the commercial preservative RNAlater was successfully used as a stabilizer to protect influenza A viral RNA for subsequent analysis. This may be of significant interest to future influenza viral research. As detailed in Chapter 2, a constellation of studies have applied real tme RT-PCR assays to verify the (+) positivity of influenza A viruses from various types of samples. The results of this dissertation study were further support of the use of RNAlater

and the application of real time RT-PCR methodologies utilizing the commonly recognized “TaqMan® principle” for water samples. As aforementioned, the nucleotide sequencing results of the real time RT-PCR amplification products validate the real time RT-PCR methodologies for pond water samples. However, the results of this dissertation study cannot fully support the use of MDCK cell line for avian influenza field research infectivity studies.

Brown et al. (2009) and others have conducted infectivity studies using MDCK cell line. This laboratory approach is also recommended by the World Health Organization (2007). However, due to the unexpected delays in preparation of MDCK cell culture and testing for hemagglutination using MDCK cells in combination with a lack of a positive influenza A virus control; the results of the hemagglutination plaque assay of this dissertation study should be interpreted with caution. Due to the unexpected delays in preparation and testing for hemagglutination using MDCK cells the results may not be truly representative of influenza A virus viability and infectivity. A positive influenza A virus control provides the desired experimental outcome for comparison against the samples. Without a positive influenza A virus control this comparison is not possible. Therefore, the results of the hemagglutination plaque assay should be interpreted with caution. The MDCK infectivity assay outcomes experienced in this dissertation are possibly of significant interest to future research of aquatic habitats and the avian influenza viruses within them.

Limitations of the Study

As aforementioned in Chapter 1, there are two threats to the external validity of this study. The first threat to external validity is centered to the theoretical framework of this dissertation. The theoretical framework for this study was based on the hypotheses proposed by Webster et al. (1992) and others that (a) migratory waterfowl are the natural reserve of influenza viruses, and (b) water-borne transmission of influenza virus occurs between migratory waterfowl and domestic waterfowl. The inclusion criteria of the study population were artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. The geographical area was the state boundaries of California. Thus, the findings of this dissertation are limited to geographical locations along the migratory flyways of the waterfowl within the boundaries of California.

As aforementioned in Chapter 4, the severe drought conditions in California resulted in a limited the number of available artificial recirculating water ponds for sampling. Furthermore, the wildfires in Northern California during the time frame of the field research and data collection made several of the planned pond water locations unsafe for travel and field research. It is reasonable the severe drought conditions limited the number of available water ponds for migratory waterfowl migration stopovers. A similar effect may be attributed to the 2014 summer wildfires in Northern California. The unsafe smoke and fire across several geographical regions are likely to have diverted migratory waterfowl migration towards safer flight paths and stopovers. These environmental conditions may have contributed to the outcomes of this study.

This study is limited to the geographical areas of California which were available for pond water sampling. These areas were defined by both state boundaries and environmental conditions, namely drought and wildfire. As is shown in the analysis of the data, the greater proportion of samples verified for influenza A virus were collected within urban counties. This increased proportion maybe due to migratory waterfowl migration seeking safer flight paths and stopovers. As observed during the field research phase of this study, the urban counties had fewer wildfires and more available pond water.

Recommendations

The purpose of this dissertation study was to investigate the aquatic virus reservoir in rural and urban communities. As such, this was a baseline water analysis study of the proportion of influenza A virus in urban and rural community settings in California. Future research should continue to investigate the aquatic virus reservoir in rural and urban communities. Alterations should be made to the ecological and geographical approach and also to the laboratory model of this dissertation to improve the focus and yielding data.

The ecological and geographical approach could be improved by narrowing the geographical area of interest and using a multicenter strategy. This dissertation research was a single-center statewide cross sectional study. Limited resources and distances of travel reduced the allocation of funds per sample in this study. A multicenter approach over a limited geographical may have a higher allocation of funds per sample. Greater funding per sample may further result in identifying influenza A viruses. Identifying

influenza A viruses by geography may improve vaccine development and biosecurity measures.

The results of this study favor water ponds in urban communities to have a higher burden of influenza A viruses than rural communities. Future research could limit sampling schemes to specific urban communities. This dissertation study was limited to collecting a single 200-ml water sample per site. It would be prudent for future studies to collect multiple water samples from a single site. Additionally, in place of targeting only for H5N1 influenza A viruses by real time RT-PCR, future studies could use universal primer sets (PanHA) for amplification and sequencing for all 16 HA subtypes as asserted by Gall, Hoffmann, Harder, Grund, and Beer (2008). A similar modification could also be used for neuraminidase (NA) subtyping. Huang, Khan, and Măndoiu (2013) asserted that quadruplicate primer pools [(N2, N6, N7); (N4, N5, N7, N8); (N3, N5, N9); and (N1, N4, N6, N9)] of specific primer sequences can effectively be used to identify and sequence all 9 NA subtypes. Influenza A viral sequencing may also be improved by modification to the laboratory model.

The additional step of agarose gel electrophoresis following conventional RT-PCR is a recommended protocol by the World Health Organization (2007) (this study used real time RT-PCR assays). Agarose gel electrophoresis is a process to identify and isolated DNA groups from a sample for the purpose of nucleotide sequencing. In the Gall et al. (2008) study, the researchers purified the PanHA RT-PCR products using agarose gels and by use of the QIAquick gel extraction kit (Qiagen®, USA) as recommended by

the manufacturer. The purified influenza viral samples were then genetically sequenced (Gall et al., 2008).

According to Gall, et al. (2008), the PanHA RT-PCR primers were designed and combined in a one-step RT-PCR to amplify a fragment of 164 to 176 bp spanning the HA_0 cleavage site of influenza A viruses of all 16 HA subtypes. A similar process was also used by the researchers of the Huang, Khan, & Măndoiu (2013) study in evaluating the sensitivity and specificity of the quadruplicate primer pools to identify and sequence all 9 NA subtypes. The agarose gel electrophoresis technique was not used in this dissertation study. Agarose gel electrophoresis technique may have improved the nucleotide sequencing results. Adapting these recommendations to a multicenter approach would improve the research model and deepen the geographical areas of interest.

Institutions of higher education with capabilities to conduct the laboratory research could form a multicenter influenza A virus surveillance consortium. The University of California system consists of 10 campuses and the California State University system consists of 23 campuses. These institutions of higher learning are located throughout the state. This dissertation research study could readily be adapted to the classroom setting. The adaptation and implimentation of this dissertation research study to the curriculum at institutions of higher learning has the potential to increase influenza A virus surveillance and monitoring at the human-animal interface, improve upon the laboratory model, increase and improve upon the influenza A virus strain library for vaccine development, and may bring greater awareness to the individual, and thus,

greater perceived susceptibility to influenza A virus infection. Greater perceived susceptibility to influenza A virus infection may lead to increased influenza vaccination rates.

As previously noted, indigenous populations in the United States have shown a disproportionately higher rate of hospitalization and death from pandemic 2009 influenza A (H1N1) virus infection. The Centers for Disease Control and Prevention (2009) reported American Indian/Alaskan Natives had H1N1 mortality rates four times higher than persons in all other racial/ethnic populations combined. The findings of this dissertation study show a greater burden of influenza A viruses in urban counties' community water ponds, therefore, further investigation into rural communities' human-animal interface is warranted to rule-out potential sources of influenza A viruses that may not have been sampled. As shown in Figure 16 and Figure 17, there are identifiable clusters of Native American Reservation in the Continental United States and California (see Table 1). There are geographical similarities to water ponds verified (+) positive for influenza A viruses (see Figure 18) and the locations of Native American Reservations in California (see Figure 17). Further investigation of water ponds proximal to Native American Reservations in California may be of significant interest to understanding the disparate outcomes H1N1 mortality rates among American Indian/Alaskan Natives. Another area of future research needs to focus on eliminating influenza A viruses or reducing influenza A virus infectivity.

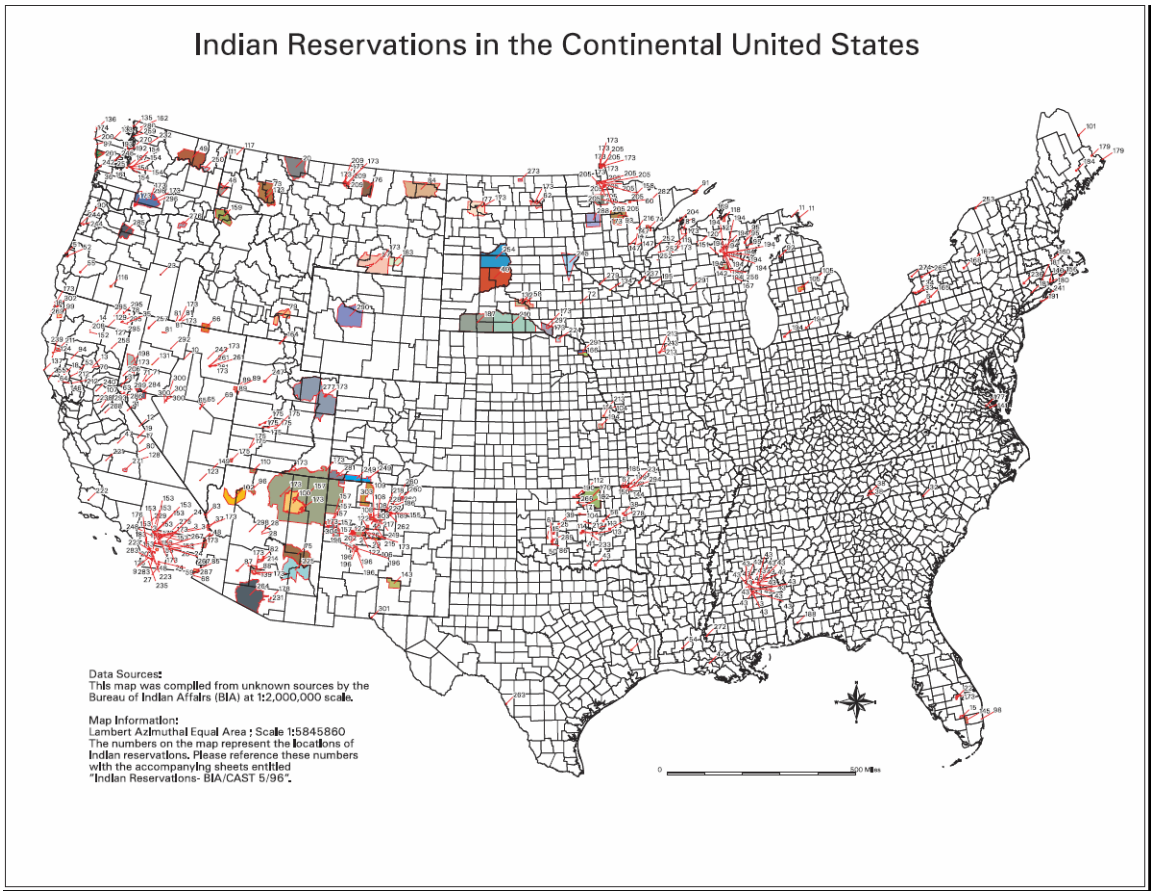

Figure 16. Native American reservations in the continental United States site map.

Table 10

Native Americans Reservations in the Continental United States Map Index

Native American Tribe and Index Number					
0 No Data	51. COOS, LOWER UMPQUA & SIUSLAW	102. HUALAPAI	153. MORONGO	204. RED CLIFF	255. STEWARTS POINT
1. ABSENTEE SHAWNEE *	52. COQUILLE *	103. INAJA	154. MUCKLESHOOT	205. RED LAKE	256. STOCKBRIDGE MUNSEE
2. ACOMA	53. CORTINA	104. IOWA *	155. NAMBE	206. RENO-SPARKS	257. SUMMIT LAKE
3. AGUA CALIENTE	54. COUSHATTA	105. ISABELLA	156. NARRAGANSETT	207. RINCON	258. SUSANVILLE
4. ALABAMA-COUSHATTA	55. COW CREEK	106. ISLETA	157. NAVAJO	208. ROARING CREEK	259. SWINOMISH
5. ALABAMA-QUASSARTE CREEKS *	56. CREEK *	107. JACKSON	158. NETT LAKE	209. ROCKY BOYS	260. TAOS
6. ALLEGANY	57. CROW	108. JEMEZ	159. NEZ PERCE	210. ROSEBUD	261. TE-MOAK
7. APACHE *	58. CROW CREEK	109. JICARILLA	160. NIPMOC-HASSANAMISCO +	211. ROUND VALLEY	262. TESUQUE
8. BAD RIVER	59. CUYAPAIPE	110. KAIBAB	161. NISQUALLY	212. RUMSEY	263. TEXAS KICKAPOO
9. BARONA RANCH	60. DEER CREEK	111. KALISPEL	162. NOOKSACK	213. SAC AND FOX #	264. TOHONO O'ODHAM
10. BATTLE MOUNTAIN	61. DELAWARE *	112. KAW *	163. NORTHERN CHEYENNE	214. SALT RIVER	265. TONAWANDA
11. BAY MILLS	62. DEVILS LAKE	113. KIALEGEE CREEK *	164. NORTHWESTERN SHOSHONE	215. SANDIA	266. TONIKAWA *
12. BENTON PAIUTE	63. DRESSLERVILLE COLONY	114. KICKAPOO *	165. OIL SPRINGS	216. SANDY LAKE	267. TORRES MARTINEZ
13. BERRY CREEK	64. DRY CREEK	115. KIOWA *	166. OMAHA	217. SANTA ANA	268. TOULUMNE
14. BIG BEND	65. DUCKWATER	116. KLAMATH *	167. ONEIDA #	218. SANTA CLARA	269. TRINDAD
15. BIG CYPRESS	66. DUCK VALLEY	117. KOOTENAI	168. ONONDAGA	219. SANTA DOMINGO	270. TULALIP
16. BIG LAGOON	67. EASTERN SHAWNEE *	118. L'ANSE	169. ONTONAGON	220. SANTA ROSA	271. TULE RIVER
17. BIG PINE	68. EAST COCOPAH	119. LAC COURTE OREILLES	170. OSAGE	221. SANTA ROSA (NORTH)	272. TUNICA-BILOXI

(table continues)

Native American Tribe and Index Number

18. BIG VALLEY	69. ELY COLONY	120. LAC DU FLAMBEAU	171. OTOE-MISSOURI *	222. SANTA YNEZ	273. TURTLE MOUNTAINS
19. BISHOP	70. ENTERPRISE	121. LAC VIEUX DESERT	172. OTTAWA *	223. SANTA YSABEL	274. TUSCARORA
20. BLACKFEET	71. FALLON	122. LAGUNA	173. OUT	224. SANTEE	275. TWENTYNINE PALMS
21. BRIDGEPORT	72. FLANDREAU INDIAN SCHOOL	123. LAS VEGAS	174. OZETTE	225. SAN CARLOS	276. UMATILLA
22. BRIGHTON	73. FLATHEAD	124. LAYTONVILLE	175. PAIUTE	226. SAN FELIPE	277. UINTAH AND OURAY
23. BURNS PAIUTE COLONY	74. FOND DU LAC	125. LA JOLLA	176. PALA	227. SAN ILDEFONSO	278. UNITED KEETOOWAH BAND OF CHEROKEE *
24. CABEZON	75. FORT APACHE	126. LA POSTA	177. PAMUNKEY +	228. SAN JUAN	279. UPPER SIOUX
25. CADDO *	76. FORT BELKNAP	127. LIKELY	178. PASCUA YAQUI	229. SAN MANUAL	280. UPPER SKAGIT
26. CAHUILLA	77. FORT BERTHOLD	128. LONE PINE	179. PASSAMAQUODDY	230. SAN PASQUAL	281. UTE MOUNTAIN
27. CAMPO	78. FORT BIDWELL	129. LOOKOUT	180. PAUCATAUK PEQUOT +	231. SAN XAVIER	282. VERMILION LAKE
28. CAMP VERDE	79. FORT HALL	130. LOS COYOTES	181. PAUGUSETT +	232. SAUK SUIATTLE	283. VIEJAS
29. CANONCITO	80. FORT INDEPENDENCE	131. LOVELOCK COLONY	182. PAWNEE *	233. SEMINOLE *	284. WALKER RIVER
30. CAPITAN GRANDE	81. FORT MCDERMITT	132. LOWER BRULE	183. PECHANGA	234. SENECA-CAYUGA *	285. WARM SPRINGS
31. CARSON	82. FORT MCDOWELL	133. LOWER ELWAH	184. PENOBSCOT	235. SEQUAN	286. WASHOE
32. CATAWBA	83. FORT MOHAVE	134. LOWER SIOUX	185. PEORIA *	236. SHAGTICOKE +	287. WEST COCOPAH
33. CATTARAUGUS	84. FORT PECK	135. LUMMI	186. PICURIS	237. SHAKOPEE	288. WHITE EARTH
34. CAYUGA *	85. FORT YUMA	136. MAKAH	187. PINE RIDGE	238. SHEEP RANCH	289. WICHITA *
35. CEDARVILLE	86. FT. SILL APACHE *	137. MANCHESTER	188. POARCH CREEK	239. SHERWOOD VALLEY	290. WIND RIVER
36. CHEHALIS	87. GILA BEND	138. MANZANITA	189. POJOAQUE	240. SHINGLE SPRING	291. WINNEBAGO #

(table continues)

Native American Tribe and Index Number

37. CHEMEHUEVI	88. GILA RIVER	139. MARICOPA	190. PONCA *	241. SHINNECOCK +	292. WINNEMUCCA
38. CHEROKEE * #	89. GOSHUTE	140. MASHANTUCKET PEQUOT	191. POOSEPATUCK +	242. SHOALWATER	293. WOODFORD INDIAN COMMUNITY
39. CHEYENNE-ARAPAHOE*	90. GRANDE RONDE	141. MATTAPONI +	192. PORT GAMBLE	243. SHOSHONE	294. WYANDOTTE *
40. CHEYENNE RIVER	91. GRAND PORTAGE	142. MENOMINEE	193. PORT MADISON	244. SILETZ	295. XL RANCH
41. CHICKASAW *	92. GRAND TRAVERSE	143. MESCALERO	194. POTAWATOMI #	245. SISSETON	296. YAKAMA
42. CHITIMACHA	93. GREATER LEECH LAKE	144. MIAMI *	195. PRAIRIE ISLE	246. SKOKOMISH	297. YANKTON
43. CHOCTAW * #	94. GRINDSTONE	145. MICCOSUKEE	196. PUERTOCITO	247. SKULL VALLEY	298. YAVAPAI
44. CITIZEN BAND OF POTAWATOMI *	95. HANNAHVILLE	146. MIDDLETOWN	197. PUYALLUP	248. SOBOBA	299. YERINGTON
45. COCHITI	96. HAVASUPAI	147. MILLE LACS	198. PYRAMID LAKE	249. SOUTHERN UTE	300. YOMBA
46. COEUR D'ALENE	97. HOH	148. MISSION	199. QUAPAW *	250. SPOKANE	301. YSLETA DEL SUR
47. COLD SPRINGS	98. HOLLYWOOD	149. MOAPA	200. QUILLAYUTE	251. SQUAXON ISLAND	302. YUROK
48. COLORADO RIVER	99. HOOPA VALLEY	150. MODOC *	201. QUINAULT	252. ST. CROIX	303. ZIA
49. COLVILLE	100. HOPI	151. MOLE LAKE	202. RAMAH	253. ST. REGIS	304. ZUNI
50. COMANCHE *	101. HOULTON MALISEETS	152. MONTGOMERY CREEK	203. RAMONA	254. STANDING ROCK	

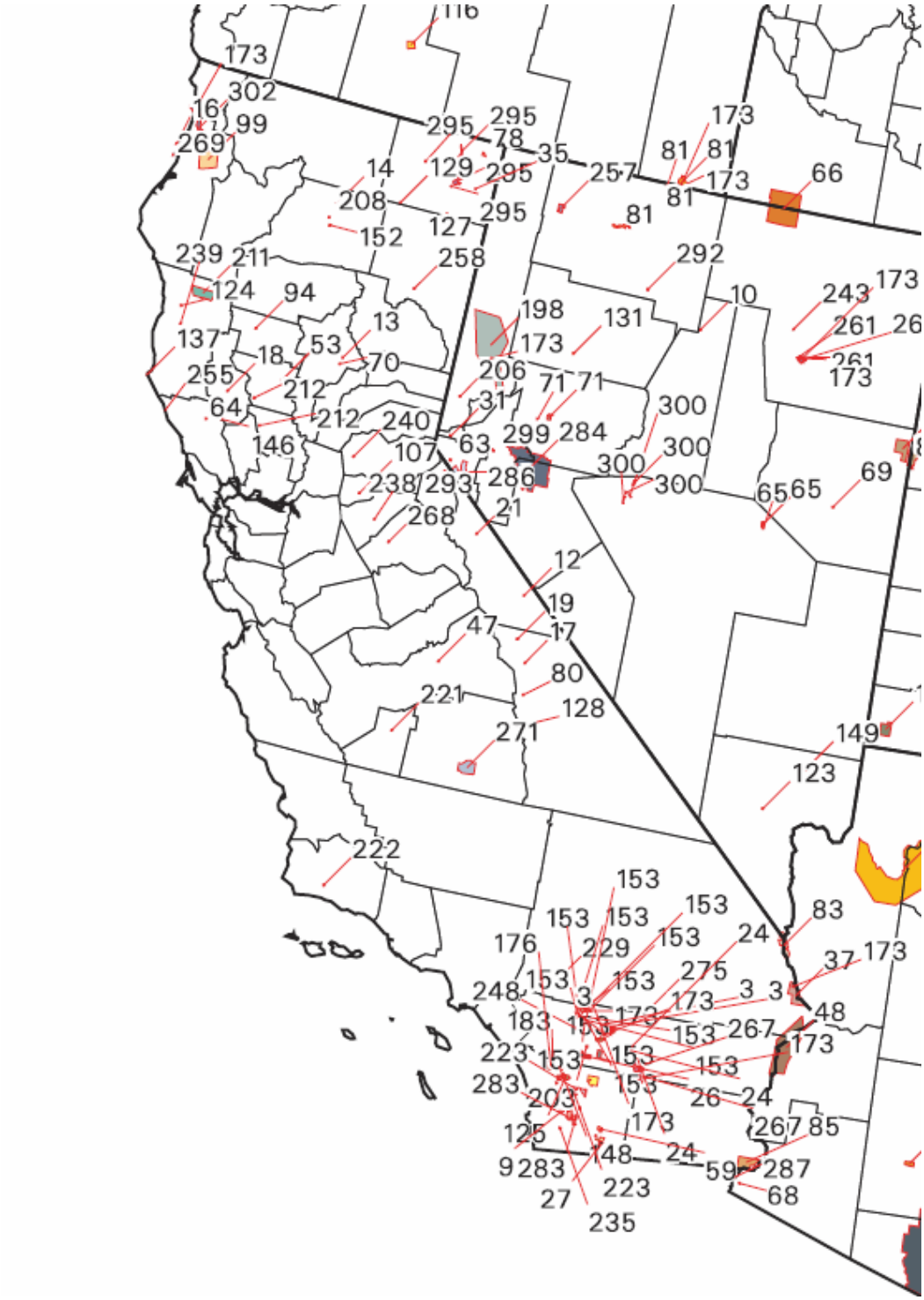

Figure 17. Native American reservations in California site map.

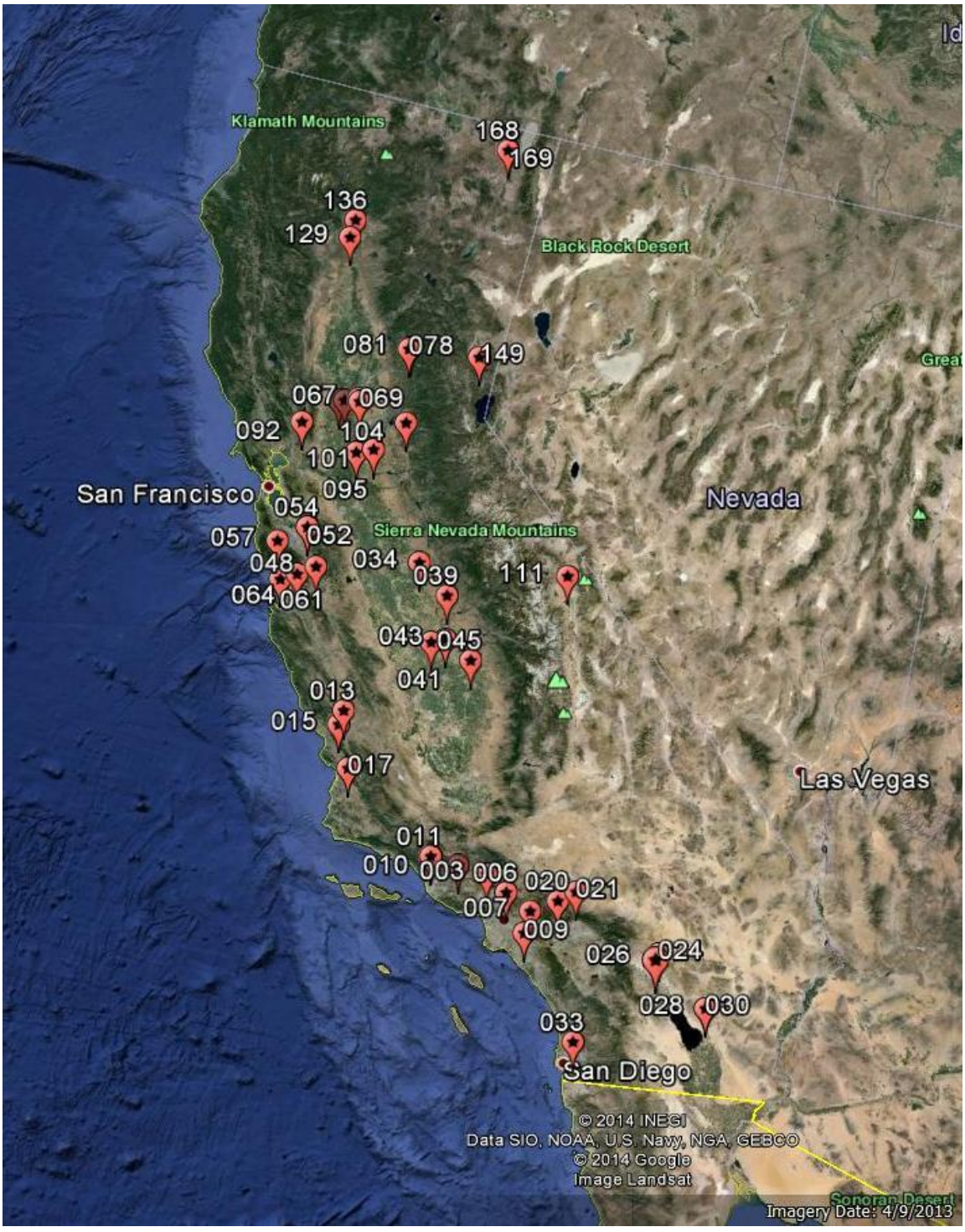

Figure 18. Statewide samples verified (+) positive for their content of influenza A virus genome.

The target population for this dissertation was artificial recirculating water ponds in the geographic locations of rural and urban Californian communities. Future studies may consider investigating methods of eliminating influenza A viruses or reducing influenza A virus infectivity in these aquatic habitats. As aforementioned, Faust et al. (2009) focused on the ability of filter-feeeding bivalves to remove influenza virus from contaminated water. The researchers were able to demonstrate that filter-feeding bivalves can remove influenza virus from contaminated water, however, the findings of the research suggest avian influenza virus is inactivated or sequestered in clam tissue after filtration (Faust et al., 2009). The introduction of bivalves to communty water ponds for the purpose of removing influenza A viruses may be ecologically unfavorable. Another method of removing influenza A viruses could be water exposure to ultraviolet electromagnetic radiation (UV light). The method of UV light to sterilize saltwater aquariums is a well known and common practice. An in-vitro investigation to the susceptibility of influenza A viruses to UV light may warrant further field research in artificial recirculating water pond settings. If successful, UV light exposure may be viewed as a biosecurity measure. These recommendations for expansion and further research are centered around positive social change.

Implications

The potential impact of this dissertation study can be recognized at the international level, the national and state level, the population level, and at the individual level. The results of this dissertation study show that artificial water ponds in communities can be sources of influenza A viruses. As sources of influenza A viruses,

the artificial water ponds in communities can be used to increase influenza A virus surveillance and monitoring at the human-animal interface, expand and improve upon the influenza A virus strain library for vaccine development, and may bring greater awareness to the individual, and thus, greater perceived susceptibility to influenza A virus infection leading to improved vaccination rates. International surveillance and monitoring of influenza viruses is a current global challenge.

At the end of 2013, seven novel variants of clade 2.3.4 HPAI H5N1 isolated from poultry in China were reported (Gu, et al., 2013). The authors of the Gu, et al. (2013) study also reported that various NA subtypes of the H5 viruses bearing the same genetic backbone of clade 2.3.4 A(H5N1) viruses had been detected in ducks, geese, quail, and chickens. Following this event, there have been two epidemic clusters of H7N9 human infections in China (World Health Organization, 2014). The World Health Organization (2014) reported there have been more than 400 cases with a case fatality rate greater than 20%. Additionally, the World Health Organization reported the source of infection is assumed to be infected poultry or contaminated environments. These two recent events alone stress the importance of international surveillance and monitoring of influenza A viruses. As aforementioned, artificial water ponds in communities can be used to increase influenza A virus surveillance and monitoring at the human-animal interface. Increased surveillance and monitoring of influenza viruses may lead to mitigating risk. Mitigating influenza risk is a challenge at the national and state levels.

Moriguchi, Onuma, and Goka (2013) asserted that species distribution models can be useful tools for predicting the distribution of pathogens. The authors of the Moriguchi,

et al. study further asserted the creation of a potential risk map for avian influenza, including the localities of infected birds, would provide a potentially useful warning of infection and also be used to suggest priority areas for surveillance. This dissertation research study model could supplement the potential risk map for avian influenza as suggested by Moriguchi, et al. by providing molecular verification of the presence of influenza A viruses. The national and state level impact of potential risk mapping and aquatic habitat surveillance for influenza A viruses would be an initiation of biosecurity measures to protect poultry industries. Surveillance for influenza A viruses in aquatic habitats in communities may also lead to positive impact at the population level.

Improving vaccine development is a positive impact at the population level. This dissertation research study as a model, may further expand the influenza A virus strain library for vaccine development. Schrauwen et al. (2014) asserted surveillance of bird and swine influenza viruses should specifically target particular mutations that render viruses more virulent or airborne-transmissible in humans. The virulence of influenza A viruses is being studied by others (Rumschlag-Booms & Rong, 2013; Schrauwen et al., 2014; Taggi, Colaiori, Loreto, and Tria, 2013). Rumschlag-Booms and Rong (2013) stated the HA protein of the influenza A viron is recognized as an important determinant for influenza virus virulence and pathogenesis (p. 2). The researchers of the Schrauwen et al. (2014) study presented a similar conclusion and also noted the polymerase proteins (PB2), PB1-F2, PA-X, NS1, and NA each are contributing virulence factors. Taggi, Colaiori, Loreto, and Tria (2013) asserted the evolutionary dynamics of human influenza A viruses have a high mutation rate that allows the viruses to escape the host's adaptive

immunity. The theorectical model proposed by Taggi, et al. highlights the challenges of developing a universal vaccine that would induce protection against all influenza subtypes (Schrauwen et al., 2014). As aforementioned, this dissertation research study as a model, may further expand the influenza A virus strain library for vaccine and theapeutics development. Therapeutic development is a positive impact at the individual level. Further, at the individual level the results of this dissertation may bring about greater awareness of influenza A virus infection.

As previously noted, Shive and Kanekar (2011) suggested the principles of the Health Belief Model can provide explanation why many people, including high risk individuals, do not receive influenza vaccination. The results of this dissertation study may bring greater awareness to the individual, and thus, greater perceived susceptibility to influenza A virus infection. The analysis of samples from artificial recirculating water ponds identified influenza A viruses. Many of the samples were collected from artificial recirculating water pond features in parks and golf courses. The decorative feature is often constructed to attract people. These decorative features may also attract both domestic and migratory waterfowl (Webster et al., 1992). As asserted by Webster et al. (1992), migratory waterfowl likely will introduce influenza A viruses to the artificial recirculating water pond enivronment from viral shedding in feces. Several of the artificial recirculating water ponds sampled were constructed with an aeration fountain producing mist and droplet formation. As is well established, influenza viruses are transmitted between individuals through droplets (e.g., coughing, sneezing). The individual who comes in contact with the droplets from the contaminated artificial

recirculating water pond may be inoculated with influenza A virus. Therefore, the (+) positive influenza A virus findings of this dissertation study may bring greater awareness at the individual level, and thus, increase the perceived susceptibility to influenza A virus infection. As implied by Shive and Kanekar, perceived susceptibility to influenza A virus infection will have a direct correlation to influenza vaccination.

Conclusion

The results of this dissertation research study show community water ponds are viable sites of influenza A viruses. Additionally, the data analysis suggests these aquatic systems may serve as reservoirs and sources of infection for both wild birds and mammals. The infectivity studies show these community water ponds to have the potential to be sites of the human-animal interface for influenza A virus infection. Additionally, this environmental baseline research study validated these community water ponds as resource sites for influenza A virus surveillance and monitoring. The materials and methods of this environmental baseline research study were built on the research of others.

This investigation successfully used the commercial preservative RNAlater (Qiagen®) as a stabilizing media for influenza A virus research. Real time RT-PCR utilizing the TaqMan® methodology also showed to be successful. Testing field research water samples with the hemagglutination MDCK plaque assay showed promise and is warranted for further investigation. The geochemical properties of pond water (pH, salinity, and temperature) could not be concluded as contributing factors to influenza A virus infectivity in water ponds. However, due to the association of the MDCK plaque

assay in this study, the geochemical properties of pond water as contributing factors to influenza A virus infectivity also warrants further investigation. Alterations should be made to the ecological and geographical approach of this research study to improve the focus and resulting data.

This research study was a single-center statewide cross sectional study. The ecological and geographical approach could be improved by narrowing the geographical area of interest and using a multicenter strategy. Institutions of higher education with capabilities to conduct the biosafety level 2 laboratory research could collaborate to form a multicenter influenza A virus surveillance consortium. This research study could readily be adapted to the classroom setting. The adaptation and implimentation of this dissertation research study to the curriculum at partnership institutions has the potential to increase statewide influenza A virus surveillance and monitoring, increase and improve upon the influenza A virus strain library at the national and international levels for vaccine development, and may bring greater awareness to the individual and society about susceptibility to influenza A virus infection.

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Appendix A: Collection Site Contact Letter

Collection Site Contact Letter

[Date]

Zin Htway
Contact Information

Dear [Data Collection Site Contact],

I am a PhD student at Walden University and I am conducting a research study entitled _____. This research involves the collection of water samples from artificial recirculating water ponds in rural and urban communities throughout California. By this letter, I am requesting your (and/or your organization's) participation in this research. Of course, your participation in the research is completely voluntary. The data collection process at your site will present no risk of harm or damage to your facility or any individuals who may be present. The potential benefits from this research include _____. Your participation in the research as well as the data derived from the samples collected from your site were kept confidential. While the description of the research in this letter has been summary in nature, I will provide the full research proposal for your information should you desire (either now or at any point in the future). If you have any questions about the proposed research please feel free to contact me (now or in the future) at _____. If you want to talk privately about your rights regarding this research, you can call Dr. Leilani Endicott. She is the Walden University representative who can discuss this with you. Her phone number is 612-312-1210. Walden University's approval number for this study is **IRB will enter approval number here** and it expires on **IRB will enter expiration date.**

If you (and/or your organization) agree to participate in the research, please sign the attached letter of cooperation and return to me. Also, you should keep a copy of the signed letter of cooperation for your files. Please feel free to contact me if you have any questions or concerns.

Sincerely yours,

Appendix B: Letter of Cooperation

Letter of Cooperation

[Date]

Data Collection Site
Contact Information

Dear Zin Htway,

Based on my review of your research proposal, I give permission for you to conduct the study entitled _____ at our site located at _____. As part of this study, I authorize you to collect water samples as described in your contact letter dated ____ and more specifically detailed in your research proposal.

We understand that our organization's responsibilities include: _____. We reserve the right to withdraw from the study at any time if our circumstances change.

I confirm that I am authorized to approve research in this setting.

I understand the data collected will remain entirely confidential and may not be provided to anyone outside of the research team without permission from the Walden University IRB.

Sincerely,
Authorization Official
Contact Information

Appendix C: Curriculum Vitae

Zin M. Htway

zin.htway@waldenu.edu / zin.htway@csuci.edu

Education:

PhD in Public Health, Epidemiology, Walden University, February 2015

MBA, Healthcare Management, Western Governor's University, 2011

Bachelor of Science in Clinical Sciences, Cytotechnology, California State University Dominguez Hills,

1992

Certificate of Completion, Los Angeles Consortium of Cytotechnology, University of California Los Angeles and California State University Dominguez Hills, 1992

Professional experience:

Walden University

Graduate Assistant, Biostatistics and Advanced Biostatistics 2012-Present

Developed and practiced Peer Mentoring program for following Quarter-based courses:

PUBH-8125 Biostatistics

PUBH-8500 Advanced Biostatistics

California State University Channel Islands 2008-Present

Lecturer

Taught the following lecture and lab courses:

Biology 426 Hematology Lecture

Biology 426 Hematology Laboratory

Biology 508 Advanced Immunology Lecture, Master's Level

Biology 508 Advanced Immunology Laboratory, Master's Level

Biology 432 Epidemiology and Environment Health Lecture

Biology 322 Cancer and Society

Biology 213 Sex, Germs, and Diseases

Los Robles Hospital and Medical Center, Thousand Oaks, California 2005-Present

Supervisor Anatomic Pathology Department

Hospital HazMat Safety Officer

Laboratory Team Leader, Ebola

Responsibilities include – Operations Manager of the Anatomic Pathology Section of the Department of Laboratory Services; which include Surgical Pathology Section, Cytology and Cytology Preparation Sections, Histology Sections, Immunohistochemistry Sections, Molecular Pathology Section, Transcription and Pathology Office, Anatomic Pathology Morgue, maintaining Department of Pathology Quality Assurance Plan, developing and maintaining relationships with other Hospital Departments through Quality Assurance and Quality Improvement, Primary liaison to various Reference Laboratories for Anatomic Pathology testing (send-out testing). Additional responsibilities include – Maintain biological waste, hazardous waste, and environmental regulatory certifications for national, state, and local jurisdictions for the three hospital system; develop and train hazmat response teams and Mass Casualty Incidence response teams.

Interscope Pathology Medical Group, Canoga Park, California 2004-2005

Managing Director of Cytology and Client Services

Responsibilities include – Managing the Cytology Department, the Cytology Preparation Department, the Fine Needle Aspiration Clinic, the DNA/HPV Laboratory, the Client Services Department, the Business Development Department, the Courier and Dispatch Department, Laboratory Receptionist(s), Webpage Development and Coordination, Coordinator for IT / IS Consultants, and Coordinator for United West Laboratories (A Clinical Pathology Laboratory affiliate).

Interscope Pathology, Canoga Park, California 2004-2004

Chief Cytotechnologist/Assistant Cytology Department Manager

Responsibilities include – Microscopic slide examination of Gynecological and Non-gynecological specimens utilizing Cytyc Imaging System and a Nikon Eclipse E400 light microscope, maintaining Quality Control and Quality Assurance protocols and logs, distributing and collecting Continuing Educational materials and certificates, reviewing and releasing of Final Diagnosis Reports for Physician Clients, coordinating workflow between Pathologists, Cytotechnologists, Courier Department and Cytology Laboratory Assistants, scheduling and managing on-call staff to maintain workflow and specimen "turn-around-time", communicating with Cytyc Corporation (vendor) for purpose maintaining Cytyc Imaging System equipment as well as equipment data analysis/performance, ensuring proper medical billing coding for collection of laboratory services.

Pathology Inc, A Medical Corporation, Torrance, California 2003-2004

Senior Cytotechnologist

Responsibilities include – Coordinating workflow between Cytology Laboratory Assistants and evening Cytotechnology Staff, reporting directly to Chief Operations Officer. Bench work included microscopic examination and diagnosis of Gynecological and Non-gynecology specimens utilizing a Nikon Eclipse E400 light microscope and maintaining Quality Control and Quality Assurance protocols and logs.

Clinical Laboratories Hawaii, Honolulu, Hawaii 2003-2003

Senior Cytotechnologist

Responsibilities include – Bench Cytotechnologist, including reviewing and consulting with Pathologists for abnormal and difficult cases, reviewing and releasing of Final Diagnosis Reports to Physician Clients.

Diagnostic Laboratory Services, Honolulu, Hawaii 1997-2003

Senior Cytotechnologist (Queen's Medical Center Pathology Laboratory)

Responsibilities include – Teaching and lecturing to Pathology Residents, Medical Students, and other Medical Professionals on a variety of Cytopathology subjects and disciplines. Presentation and distribution of Continuing Educational material to staff, training new staff, working, coordinating, developing, and problem solving between Hospital Staff, Laboratory Staff, and Pathology Staff. Bench Cytology work included

microscopic examination and diagnosis of Gynecological and Non-gynecological specimens utilizing Olympus Bmax light microscope and Neopath Imaging Analyzer, maintaining Quality Control and Quality Assurance protocols and logs, reviewing and releasing of Final Diagnosis Reports to Physician Clients.

Huntington Memorial Hospital, Pasadena, California 1992-1997

Senior Cytotechnologist

Responsibilities include –Assisting Radiologists and Pathologists during Fine Needle Aspiration Biopsy (FNAB) procedures for the collection of specimens, coordinating between intra-hospital departments for correct and complete patient information. Bench Cytology work included microscopic examination and diagnosis of Gynecological and Non-gynecological specimens utilizing light microscopy, microscopic glass slide preparation of specimens for analysis and maintaining Quality Control and Quality Assurance protocols and logs, presentation and distribution of Continuing Education material to staff, reviewing and releasing of Final Diagnosis Reports to Physician Clients.

Metwest Clinical Laboratories, Tarzana, California 1988-1992

Medical Laboratory Assistant

Responsibilities include – Processing all blood serum specimens for Laboratory Chemistry Tests requested utilizing the Olympus AU5600 Chemistry Analyzer (four units). Operating, maintaining, troubleshooting, and problem solving the Olympus AU5600 instruments, maintaining and recording all Chemistry Department Quality

Control and Quality Assurance data and logs, reviewing and releasing of Laboratory Chemistry results (under the Supervision of a Medical Technologist), coordinating and reporting of department issues with the Chemistry department Supervisor (off-site).

Beverly Enterprises – Rush Pharmacy, Camarillo, California 1985-1988

Pharmacy Assistant

Responsibilities include – Ordering prescriptions, packaging prescriptions, and presenting prescriptions to the Pharmacist for approval and release, maintaining patient databases, ordering medications for inventory levels, occasional delivery of medication to patients, scheduling of Pharmacy Assistant staff and coordination with Courier Department for delivery services.

Consulting and per diem experience:

Impact Health Biometric Testing, Inc. 2013 to present

King of Prussia, PA

Scientific Consultant

Walden University 2013 to 2014

Minneapolis, MN

Graduate Assistant/Peer Mentor Advanced Biostatistics and Biostatistics

TOPA Diagnostics 2009 to present

Thousand Oaks, CA

Scientific Consultant

Visalia Pathology Medical Group 2009 to present

Visalia, CA

Scientific Consultant

Medical Group Pathology Laboratory/Pacific Diagnostic Laboratories 2005 to 2011

Santa Barbara, CA

Per diem Cytotechnologist

Civic Involvement:

Board of Directors, South Eastern Ventura County YMCA 2009 to 2010

Executive Board, Earths Magnet Elementary School for Science and Technology 2009 to 2010

Committee Chairperson, Habitat for Humanity-LRHMC, Thousand Oaks Project *Build a Hospital; Build a Home* 2009 to 2011

Professional Organizations:

California Association of Cytotechnology (CAC)

American Society of Clinical Pathology (ASCP)

International Academy of Cytology (IAC)

Licensure:

ASCP: CT-9892

IAC: CT (IAC) 7549

California DHS: 0076

State of Hawaii DHS 3972

Certifications:

Department of Homeland Security, FEMA, Center for Domestic Preparedness: Hospital Emergency Response Training for Mass Casualty Incidents 2014

Department of Homeland Security, FEMA, Center for Domestic Preparedness: Hospital Emergency Response Training for Mass Casualty Incidents, Train the Trainer 2014