MOLECULAR EPIDEMIOLOGY OF SEASONAL AND PANDEMIC INFLUENZA A (H1N1) IN HAWAI‘I

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DEDICATION PAGE

Dedicated to my mother, June D.M. Gorgonio, whose unwavering support of my education throughout the past 22 years is the primary reason I have been able to dream so big, and come so far.
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ABSTRACT

Influenza is a viral infection causing seasonal outbreaks, periodic epidemics and global pandemics in humans, the latest being the 2009 pandemic. The State of Hawai‘i is particularly vulnerable to the spread of influenza due to its unique geographic position in the Pacific Ocean with heavily trafficked passenger and freight patterns. By combining epidemiological data on case occurrences with their laboratory-derived viral sequences, we are able to trace viral strain origins based on phylogenetic relationships between isolates.

In collaboration with the Hawai‘i Department of Health State Laboratories Division, we present a study in which seasonal, or pandemic, H1N1 influenza A viral isolates collected from infected individuals in Hawai‘i were extracted, hemagglutinin and neuraminidase genes were amplified and sequenced, and examined for evolutionary relationships and spatio-temporal patterns. Implications of molecular data are also supported by epidemiologic information and statistical support of summary transmission data. Phylogenetic analysis suggests that Hawai‘i acts as both a source and sink population for type A influenza virus: in some instances Hawai‘i isolates represented the earliest instance of a strain subsequently seen elsewhere; in other instances Hawai‘i isolates clustered with strains observed earlier in other countries or geographic regions.

Through the continued usage of molecular methods, we hope to develop an improved understanding of influenza dynamics in Hawai‘i. Targeting an area of geographic importance additionally assists in depicting how location and population distribution play a role in the spread of infectious disease. Enhanced comprehension as a result of these analyses may help to improve efficiency and effectiveness of preparation and response efforts, and reduce the impact of influenza on Hawai‘i and the continental United States.
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CHAPTER 1
Review of Influenza Transmission in the Pacific

Abstract

Influenza viruses and their transmission patterns throughout Europe and the continental United States have been widely investigated. However, recent studies have proposed many influenza viral strains may originate in Southeast Asia. Others have implicated the tropical regions of the globe as potential sources of periodic viral outbreaks in temperate regions, which exhibit stronger seasonal variations. The Pacific islands, some more than others, have served as primary gateways between North America and both tropical and temperate parts of Asia for over a century. Thus it has become increasingly important to investigate viral transmission in, and through, the Pacific region.

In this chapter I discuss global influenza transmission patterns, particularly with respect to the 2009 H1N1 influenza pandemic, its evolutionary dynamics, animal reservoirs and epidemiologic spread. I additionally discuss the challenges faced in influenza control and prevention, from the evolution of antiviral resistant variants to the origination of novel strains, which require a global approach to influenza molecular surveillance, to prevent and address influenza outbreaks.

Introduction

Although we have had surveillance in human populations across the globe for many years, a critical review of circumstances surrounding the emergence of 2009 pandemic influenza virus suggests more robust surveillance is necessary, and should also include other host species, specifically, avian and swine populations. Recent studies have proposed major antigenic shifts in influenza viruses may have frequently originated in Southeast Asia (Russell et al., 2008), whereas other studies have suggested tropical regions of the globe as potential sources of viruses from which periodic, seasonal outbreaks of influenza in temperate regions could be initiated, which exhibit stronger seasonal variations than their tropical counterparts (Rambaut et al., 2008). Because the Pacific islands lie at a gateway between tropical and temperate regions, and between Asia and North America, they may play a critical role as a large tropical source of virus for
periodic seasonal epidemics in temperate regions, and potential arena for recombination of different strains, fostered by agricultural practices. In this chapter I explore the current understanding of influenza viruses and their transmission patterns, specifically the 2009 pandemic H1N1 virus, the challenges facing influenza control, and the potential role of the Pacific region in global influenza transmission patterns.

**Viral Characteristics**

Influenza viruses belong to the family Orthomyxoviridae. There are three general influenza types: A, B, and C. Influenza A (IVA) has the highest impact in terms of human morbidity and mortality, and has been the most extensively studied of the three types. Influenza A classically has an asymptomatic and symptomatic avian reservoir, but viral subtypes are also capable of infecting a wide variety of hosts, including humans. Viral strains are transmitted from birds to an intermediate host, such as a pig, where co-infection of influenza viruses can result in a “mixing vessel,” which may then produce a new strain by reassortment of genome segments. In humans, influenza is a highly contagious respiratory infection with a short incubation period of 1 to 4 days, and attack rates of 10-30% in all regions of the world. Symptoms generally last for 3 to 5 days and mortality rates for seasonal influenza are generally low, but can be as high as 60% in pandemic situations, or 100% as demonstrated by some Alaskan villages completely decimated by 1918 influenza (Nelson, 2005; Khiabanian et al., 2009).

Influenza viruses are enveloped viruses with a negative sense RNA genome totaling 12 to 15 kb in length. The genome is divided into eight different segments encoding 11 genes: HA (hemagglutinin), NA (neuraminidase), NP (nucleoprotein), M1 (matrix), M2 (ion channel), PB1, PB2 and PA, (polymerases), NS1 and NS2 (nonstructural proteins). The PB1, PB2, and PA gene products form a complex that functions as the RNA-dependent RNA transcriptase. The HA and NA glycoproteins are the major antigens against which host protective humoral immune responses are directed. The HA protein, in particular, is recognized as an important factor in strain virulence. It is approximately 1800 bp in length, and includes a highly conserved binding site that is surrounded by five specific antigenic epitopes that can manifest rapid changes through time. Because of the antigenic significance of the HA and NA, influenza strains are
named for which recognized forms of these gene products they exhibit serologically. Sixteen HA subtypes (H1-H16) and nine NA subtypes (N1-N9) are currently recognized in birds, but only 3 HAs (H1-H3) and 2 NAs (N1-N2) have been associated with pandemic and epidemic human influenza A to date. Regular outbreaks and epidemics circulate seasonally through populations around the globe (Nelson, 2005).

The Role of Evolution in Influenza Viruses

Influenza viruses are capable of rapid evolution through both genetic mutation and reassortment, giving rise to new strains and an ability to cause epidemics, evade herd immunity and vaccines, and evolve resistance to antivirals. Previous estimates place its rate of evolution among the fastest for RNA viruses, at $1.8 \times 10^{-3}$ substitutions per nucleotide site per year (Jenkins et al., 2002). Changes in the subtype combination and sequence of the two antigenic proteins, HA and NA, occurs periodically and is denoted as antigenic shift or antigenic drift, respectively. Both are evolutionary mechanisms through which influenza viruses introduce novelty and are able to escape host immune defenses. Antigenic shift is a major change in the protein due to reassortment of the eight gene segments during co-infection, (often in swine), which leads to the generation of a new influenza subtype, and has the potential to cause a pandemic. This recombination of genetic segments between different viruses, can involve the exchange of any segment, but segments of the greatest immunological significance are the hemagglutinin or neuraminidase. Conversely, genetic drift is the result of the accumulation of relatively minor changes over time due to point mutations throughout the genome, even within the same subtype. Drift can result in smaller scale influenza activity. Both shift and drift refer to changes in the influenza virus such that the immune system may either be slow to recognize and respond adequately (as in the case of antigenic drift), or may never recognize it: the new virus subtype appears as a primary viral exposure and evades all previously developed host defenses to influenza (as in the case of antigenic shift).

The mutations in the nucleotide sequence and other genetic changes in influenza viruses over time provide insight into immune evasion, reassortment, and provides a record with which to trace the movement of influenza between different populations and geographical areas. This record is revealed through the application of molecular-based
methods, including polymerase chain reactions (PCR), RNA/DNA sequencing and phylogenetic analysis. Studies show that, although some changes in the virus genomes occur randomly, there is evidence for increased genetic variability in certain domains. An investigation by Bush et al. (1999) found 18 codons of the HA1 domain in the hemagglutinin segment of the H3 influenza subtype were under increased genetic variability. The study showed viral lineages with the greatest number of mutations in more genetically variable codons were the progenitors of future H3 lineages in 9 of the 11 most recent influenza seasons. Furthermore, the codons under increased variability were associated with antibody or receptor-binding sites (Bush et al., 1999), suggesting selection pressures for immune escape or more efficient host cell entry, respectively.

Since RNA segments of the influenza virus can recombine, and because different genes can be under different evolutionary pressures related in part to their specific functions, it is important to examine multiple segments for a more accurate phylogenetic history of the viruses. Bragstad and colleagues (2008) studied the sequences of 234 influenza isolates of human H3N2, H1N1 and H1N2 circulating in Denmark between 1999 and 2006, and included full genome analysis of 24 isolates. Overall, they found the hemagglutinin segment of H3N2 viruses formed seasonal phylogenetic clusters, but different lineages were also found to co-circulate within the same season. They reported viral evolution seemed to be influenced more by small jumps in genetic distance, rather than constant drift. Whole-genome analysis allowed them to detect reassortment between lineages from H3N2 viruses circulating through multiple seasons. In addition to observed changes clustered in antigenic sites of hemagglutinin of H3N2, similar to Bush et al. (2009), Bragstad et al. (2008) reported substitutions in the CTL-isotopes of segments NA, PA, M1, NS1, and especially NP. In general, Bragstad’s findings suggested a more precise knowledge of circulating influenza viruses through complete genome analysis may lead to a more comprehensive picture of the evolution and migration of viruses, ultimately forecasting future strains for more appropriate vaccine composition.

The consequences of reassortment in the epidemiology and evolution of influenza was further illustrated by Holmes and colleagues (2005) in their phylogenetic analysis of 156 complete genomes of human H3N2 viruses collected from New York State between 1999 and 2004. Multiple clades co-circulated at different frequencies and phylogenetic
incongruences for individual gene segments indicated multiple reassortment events. For example, one clade of H3N2 present since 2000 provided the hemagglutinin segment for all H3N2 sampled after the 2002 to 2003 influenza season. This reassortment event likely provided the progenitors, antigenically variant influenza strains that sparked the 2003 to 2004 epidemic influenza season. Interestingly, even with the same hemagglutinin, phylogenetically distinct lineages continued to co-circulate. Overall, data from this large-scale analysis demonstrated multiple lineages can co-circulate, persist and reassort in epidemiologically significant ways, and underscored the importance of broad geographic and genetic (multi-segment) surveys of influenza virus (Holmes et al., 2005).

Pandemic 2009 H1N1 Influenza

The benefit of broad geographic and genetic surveillance was exemplified in the early identification of the 2009 H1N1 influenza A pandemic beginning in Mexico in March of that year. The pandemic strain was the result of a quadruple reassortment event that included North American and Eurasian swine lineages, conferring a high degree of infectiousness to a poorly transmitting swine strain. The recombinant rapidly swept throughout the world, and galvanized international health agencies into control measures. The World Health Organization (WHO) declared a pandemic by June 2009, and by December 2009 pandemic strain influenza cases were being reported in more than 208 countries. The hemagglutinin component of the seasonal influenza vaccine was found to be ineffective against the pandemic strain, and over the course of several months, a new, separate vaccine was developed and administered to address the genetic differences (Bansal et al., 2010).

United States national data on laboratory-confirmed 2009 pandemic H1N1 collected between April 17 and July 23, 2009 indicate a mortality rate of 0.12 deaths per 100,000 people, (95% confidence interval 0.08 – 0.16), with a total of 377 pandemic-associated deaths. Influenza activity varied by locality with the highest mortality rates in Hawai‘i (0.62 deaths per 100,000), New York City (0.65 deaths per 100,000) and Utah (0.58 deaths per 100,000). Of the total pandemic-related deaths, 76% occurred in people age 18 to 65, and only 9% of deaths were reported in people over 65 years old. This is in sharp contrast to seasonal influenza where over 90% of influenza related mortality is in
the over 65 population. However, the mortality rate of the 2009 pandemic influenza was generally lower, and the majority of deaths (78%) occurred in individuals with underlying medical conditions (Fowlkes et al., 2011).

The pandemic influenza A 2009 H1N1 virus was antigenically similar to the influenza strain responsible for the 1918 influenza pandemic (Pan et al., 2010). In a study comparing protein sequences from the 2009 pandemic H1N1 to strains causing other pandemics, and to viruses isolated from human, avian and swine populations, host-specific genomic signatures of the 2009 pandemic H1N1, identified as primarily swine-like, were confirmed as highly similar to those of the 1918 pandemic H1N1 virus. It was also antigenically distinct from seasonal H1N1 influenza strains. A lack of similarity between the 2009 pandemic influenza and its nearest H1N1 viral relatives on a phylogenetic tree indicated gene segments may have circulated undetected for some period prior to the outbreak (Garten et al., 2009).

The 2009 pandemic H1N1 strain exhibited relatively low levels of genetic diversity, which suggested that its introduction into humans was a recent single reassortment event or the result of multiple reassortments of similar viral strains (Pan et al., 2010) that were circulating in swine, recombining therein several months or even several years before it emerged to cause the human outbreak (Smith et al., 2009a). Domestic pigs have always clearly been critical in influenza recombination and emergence (Smith et al., 2009a); all three pandemics of the twentieth century (1918 H1N1, 1957 H2N2 and 1968 H3N2 viral pandemics) were generated by a series of multiple reassortment events (antigenic shifts) in swine or humans which seem to have emerged over a period of years prior to pandemic recognition (Smith et al., 2009b).

Low genetic diversity in emergent strains could also be explained by a selective sweep fixing beneficial amino acid substitutions that then rapidly spread. Bhoumik and Hughes (2010) show the pandemic H1N1 may have resulted from the spread of recently derived hemagglutinin through a population of ancient, and more diverse neuraminidase segments, a phenomenon not observed in H3N2, seasonal H1N1 or H5N1 viral strains. Low genetic diversity was also observed in the polymerase basic protein 1 (PB1) segment, which suggested a similar reassortment event. Low genetic diversity in more than one gene has been a recurring feature of newly emerged influenza A pandemics,
suggesting that vaccine development early in pandemic periods could minimize mutation accumulation in viral genes of low initial variability (Bhoumik & Hughes, 2010). During the expansion of the 2009 pandemic H1N1 (pandemic alert elevated from phase four to six), one signature residue mutation (found in the NP segment), and four non-signature residues (one each in the HA and NS1 segments, and two in the NA segment) became fixed. All but one of these mutations, (one of those in the NA segment), were located in viral functional domains, suggesting they may have had roles in further human adaptation and virulence (Pan et al., 2010).

The fine-scale temporal and genetic patterns of 2009 pandemic H1N1 virus expansion in the United States through spring and fall peaks revealed distinct spatial heterogeneities associated with the first pandemic wave (March to July 2009) in Texas, Wisconsin and New York (Nelson et al., 2010). While no specific pandemic viral lineage dominated in Houston, Texas where there were relatively few cases, Milwaukee, Wisconsin, and New York City experienced major outbreaks dominated by different viral lineages derived from founding strains. Throughout the second pandemic wave that began in August 2009, all three locales were dominated by the same viral lineage, from New York City. Viral migration amongst spatially distinct populations followed from the amplification of a single viral lineage that was dominant early on in one of the world’s largest international travel hubs (Nelson et al., 2010).

Similarly, Hawai‘i and other Pacific island regions represent important travel hubs and destinations, and could play a critical role in the spread and recombination of dominant viral lineages. Nineteen Pacific islands countries and territories (excluding Hawai‘i) experienced the first 2009 pandemic H1N1 influenza reported case in June 2009 in French Polynesia (Kool, et al. 2013). Among them that reported infections, there were 1972 confirmed cases, which peaked in August 2009. There were 21 deaths of confirmed pandemic H1N1 reported in seven Pacific island countries and territories. Most pandemic-related deaths were classified as being associated with respiratory distress syndrome, multiple-organ failure, or both; many were related to pre-existing conditions. Pandemic preparation and outbreak response in these areas were facilitated by multiple partners, including local governments and the World Health Organization, yet there was a significant impact in terms of morbidity and mortality, consistent with other indigenous
and low-resource settings. Tokelau and Pitcairn were the only jurisdictions to have remained free of the pandemic strain; Tokelau specifically instituted a strict maritime quarantine (Kool et al., 2013). To fully understand the role of Pacific islands in the dissemination of influenza, an examination of influenza sources linked by travel from temperate and tropical Asia and the Americas is needed.

**Influenza Transmission Patterns in Asia & North America**

The spatio-temporal patterns of influenza transmission are critical to understanding viral emergence, evolution, and spread. Some studies (e.g., Viboud & Simonsen, 2012 & Coburn et al., 2009) have used both modeling and phylogenetics to time and track the spread of influenza viruses, which in the temperate zone have largely been associated with seasonal drivers. Influenza type H3N2, which has the highest rate of evolution among the three primary human influenza serotypes, with antigenically distinct strains emerging every 2 to 5 years, took an average of 5.2 weeks (range 2.7 to 8.4 weeks) to spread across the United States (U.S.) in a non-pandemic transmission season, according to models based on 1972 to 2002 data (Viboud & Simonsen, 2012). Across seasons, state epidemics were more synchronized than expected based on seasonality alone, with the most populous states more synchronized than less populated states. Modeling suggested these patterns might be correlated with adult workflow, implying a key role for adults in the regional dissemination of infection and a larger role for children in driving the local spread of the virus. Viboud and Simonsen (2012) also found a national outbreak does not gain momentum until the epidemic reaches a well-connected state for distribution. Historically, the influenza season starts in California more often than any other state, which is consistent with its high population size and connectivity, particularly internationally. Preferred destinations to Asia and Australia may have important influences on intercontinental influenza spread. Hawai‘i is similarly well connected internationally, particularly with travelers to/from Asia.

The spread of viruses is related not only to host population dynamics, but also to intrinsic properties of the virus that can be affected by natural selection and recombination. Basic reproduction number ($R_0$) is a measure of a virus’s epidemic potential and is defined as the average number of new cases generated by one case in a
susceptible population during the time the case is infectious. An epidemic is likely to occur if $R_0$ is greater than 1, whereas transmission will wane (i.e., transmission will dissipate) if $R_0$ is less than 1. $R_0$ for novel H1N1 influenza epidemics has been estimated at 1.4 to 1.6, below $R_0$ estimates for the 1918 pandemic strain (mean of 2, range 1.4 to 2.8) but greater than seasonal strains (mean of 1.3, range 0.9 to 2.1) (Coburn et al., 2009).

In a full genome study of 284 H1N1 and 69 H3N2 influenza isolates collected across the continental U.S. from 2006 – 2007, multiple clades of both H1N1 and H3N2 had entered and co-circulated throughout the U.S. with no clear pattern of spatial spread, but with evidence of reassortment leading to the emergence of an H3N2 clade with re-acquired sensitivity to adamantine antivirals, and an antigenically distinct H1N1 variant that went on to global dominance in the following influenza season (Nelson et al., 2008). These findings demonstrated a complex pattern of spatial spread and virus evolution through natural selection, migration, and reassortment that ultimately provided insight on how global epidemiological dynamics were shaped.

**Influenza in a Tropical Environment**

Information regarding influenza transmission in tropical regions is sparse in comparison to temperate regions (Leo et al., 2009; Hampson et al., 1999), but may play a critical role in global transmission patterns (Russell et al., 2008). In the tropics, influenza transmission occurs year round, but may sometimes be associated with two epidemic peaks corresponding to rainy seasons (Leo et al., 2009).

In Thailand, investigators reported 11% of individuals hospitalized with pneumonia were influenza-positive during the study period of September 2003 and August 2004 for an annual estimated incidence of 18-111/100,000 people. Additionally, influenza was confirmed in 23% of outpatients with an annual estimated incidence of 1420/100,000 people. With this information, it was determined that influenza virus placed a burden of between US$ 23.4 and US$ 62.9 million on Thailand and also resulted in substantial productivity losses (Simmerman et al., 2006).

Investigators in Singapore found influenza between 1996 and 2003 was associated with an annual death rate from all causes of 14.8 (95% CI 9.8 - 19.8) per 100,000 person-years, a rate comparable to that of the United States and Hong Kong. Researchers also
reported 6.5% of all pneumonia deaths in Singapore were attributable to influenza virus, with elderly populations being disproportionately affected (Chow et al., 2006). Also of concern throughout 2008 was the discovery that, although H3N2 was the dominant circulating influenza strain in Singapore, a certain mutation (His275Y) conferred oseltamivir resistance in approximately 80% of H1N1 influenza present (Leo et al., 2009).

In a separate study the Singapore hospital Tan Tock Seng was the designated screening facility for swine-origin H1N1, and investigated 300 febrile travelers with respiratory symptoms from affected countries between April 27 and May 24, 2009. Results indicated 24% (72) of the travelers were infected with H3N2, 1.6% (5) had seasonal H1N1 and 2.7% (8) were positive for influenza B following emergence of 2009 pandemic H1N1 in the region. In general, public health policy and infrastructure for vaccination in the tropics is deficient. The disconcerting presence of oseltamivir resistance in seasonal H1N1, which may undermine pandemic preparation efforts that focus on antiviral stockpiling, leaves the tropics with many challenges (Leo et al., 2009).

The tropical climate may also play a significant role in suppressing influenza transmission through this vulnerable region due to relatively high humidity and temperatures, compared to temperate areas. In a longitudinal weather analysis conducted between January 1973 and December 2002 in 359 urban U.S. counties, Barreca and colleagues (2012) found low humidity was an especially critical determinant of observed influenza mortality, even after controlling for temperature. Specifically, an absolute humidity less than 6 gram of water vapor per kilogram of air was associated with increased influenza mortality. Furthermore, model predictions suggested approximately half of the average seasonal differences in U.S. influenza mortality may be explained by seasonal differences in absolute humidity alone. In summary, low humidity was associated with increased influenza viral transmission, and subsequently increased influenza-associated mortality. Temperature was also shown to modestly influence transmission and subsequent mortality, but the results were less robust and required a more complex model (Barreca et al., 2012).

These findings were further supported by Shaman and Kohn (2008), as well as by a study performed by Lowen and colleagues (2007) using guinea pigs as model hosts.
Results found the aerosol spread (transmission) of influenza virus was dependent on both ambient relative humidity and temperature, favoring cold and dry environments. Further investigation implied the effects of humidity act largely at the level of the viral particle. Additionally, the duration of peak viral shedding in infected guinea pigs housed in colder environments (5º C) was found to be approximately 40 hours longer than animals housed at warmer temperatures (20º C). Immunologically, innate responses proved comparable between animals housed in warmer and colder environments, suggesting lower temperatures do not contribute to impairment of the innate immune system.

Although the aforementioned studies suggested a warm, humid climate may confer some degree of protection, specific data from the tropics is lacking. It is also possible this warm climate may result in other exposure risks as compared to more temperate environments, especially pertaining to agricultural practices conducive to domesticated animals and human populations existing in close proximity to each other.

Human and animal living conditions, specifically those relating to domesticated poultry and swine, throughout the tropical regions such as Asia-Pacific and Southeast Asian countries, play an important role in influenza transmission. For example, H3N2 variant viral strains in humans have been associated with close contact between domestic swine and humans (Centers for Disease Control and Prevention [CDC], 2012), and highly pathogenic H5N1 avian influenza in humans has been strongly correlated with close proximity of humans and domestic poultry (Sultana et al., 2012). A systematic review conducted by Conan and colleagues (2012) reported poultry was an important sector of animal production, and backyard flocks constituted a large majority of poultry production, over 80% of the total poultry population in some areas. This is especially true in developing countries where owners of backyard flocks reported using the poultry both to meet household food demands and as a source of income. However, among an analysis of 62 articles related to safe poultry practices, only one article was found to address backyard flocks, suggesting a need for an increased focus on this agricultural practice in relation to influenza risk (Conan et al., 2012). Separately, an article by Levings (2011) emphasized the need for improvements in influenza surveillance among humans and animals. The importance of enhanced integration and communication between human and zoonotic surveillance professionals was also highlighted to promote efficient disease
preparation and response in the United States. Levings further discussed the promotion of the “one health,” which encompassed veterinary, public, wildlife and environmental health in an all-hazard approach to influenza surveillance, preparation, and response.

The importance of influenza surveillance in both human and animal populations, as well as integration of the two, was further reinforced by a study performed in Egypt, where integrated surveillance data was promoted for improved prediction of disease risk, environmental risk determinants, and identification of regional factors affecting risk. Study findings indicated human cases of H5N1 were temporally associated in a statistically significant manner with outbreaks in poultry populations. A multivariate risk model further correlated low temperatures, rural environments, high poultry density and recent avian outbreaks with an increased risk of highly pathogenic avian influenza cases in humans (Rabinowitz et al., 2012).

Agricultural practices utilized in Egypt were similar to those of Southeast Asia and the Asia-Pacific region. In Bangladesh, a 2008 study of two rural villages found backyard poultry flocks were present in 92% of households and frequent contact with both poultry and poultry feces during feeding and cleaning. In one village, 85% of households further reported poultry living in bedrooms with family members. In both villages, poultry were commonly reported to scavenge for food among household kitchen and living areas, as well as to drink and bathe in the same water used by humans for bathing and washing of utensils and clothing (Sultana et al., 2012). Close proximity of domesticated animals and their human owners, as described in Bangladesh, is also common throughout the Southeast and Pacific regions.

Challenges in Influenza Control

Today we face numerous challenges in the control of influenza viruses, including the rapid globalization of humans and animals, growing commercial domestication of alternative hosts that provide opportunities for recombination and evolution of influenza, and the widespread evolution of vaccine and antiviral evasion.

Among the most striking evolutionary changes observed in influenza is the emergence of drug resistance through the strong selection pressure placed on the viruses from over use of antiviral compounds, particularly in connection to agricultural practices.
There are currently two pharmaceutical classes of drugs available for chemoprophylaxis and treatment of influenza. The first class is neuraminidase inhibitors, which prevent the release of mature progeny viruses from an infected cell, and thereby reduce the number of newly infected cells. Neuraminidase inhibitors are available in the form of oseltamivir (brand name Tamiflu) or zanamivir (brand name Relenza). The second class is M2 protein inhibitors, which target the M2 protein needed for efficient un-coating of influenza virus within the cell, is available as amantadine (under multiple brand names) or rimantadine (brand name Flumandine).

The efficacy of chemoprophylaxis is reportedly comparable to vaccine efficacy. In general, vaccines are approximately 80% effective in preventing laboratory-confirmed illness, however, it is less effective in elderly populations, and true vaccine efficacy is strongly dependent on how well the vaccine strains match current circulating viruses and consequently varies by influenza season. Comparatively, prophylaxis with amantadine was roughly between 80% and 90% effective against influenza in the inter-pandemic period, however, it was predicted to be only 60% to 70% effective against pandemic viruses. Oseltamivir was reportedly 82% effective and zanamivir is 84% effective (Regoes & Bonhoeffer, 2006).

Drug resistance at high levels is typically conferred to both types of inhibitors by single amino acid substitutions in the M2 protein or neuraminidase viral segment, respectively. Furthermore, mutations in M2 protein inhibitors confer full cross-resistance to both amantadine and rimantadine, whereas several mutations to neuraminidase inhibitors are drug specific. There are two key factors, which affect the epidemiology of drug resistance in influenza viruses. The first factor is the rate at which drug treatment generates new resistance. Resistant isolates were found in roughly 18% of oseltamivir-treated children. The second factor is the change in fitness associated with drug resistant mutations. The fitness costs among M2 inhibitor-resistant mutants have been identified as relatively low, and mutants have demonstrated easy human transmission. Conversely, most mutations associated with resistance to neuraminidase inhibitors have typically been associated with high fitness costs. However, for these mutants, fitness costs for resistance do not need to be high, since compensatory mutations could arise to restore fitness without affecting drug resistance (Regoes & Bonhoeffer, 2006).
For M2 inhibitors, widespread resistance was identified in 2005, prompting the CDC to recommend against use of the drug in 2006 (CDC 2006). One of the major factors identified as leading to high levels of resistance to M2 inhibitors was the addition of M2 inhibitors in domesticated chicken feed, particularly throughout Asia, during the emerged threat of H5N1 avian influenza in the late 1990s and early 2000s (Parry 2005; Ilyushina, Govorkova, & Webster, 2005).

Prevalence of drug resistance has also grown substantially over the past several years. In 2004, 12% of globally collected isolates demonstrated resistance to M2 inhibitors, with between 70 and 74% of those resistant isolates collected in China and Hong Kong. However, in the beginning of the 2005-2006 influenza season 92% of all H3N2 isolates tested in the United States were found to be M2 resistant. Modeling investigation by Regoes and Bonhoeffer (2006) demonstrated when antiviral treatment was used for symptomatic individuals only, there was a relatively small fraction of newly developed resistant cases and slow increases in relative fitness. In comparison, when prophylaxis was used in addition to treatment there was a substantial increase in resistance in terms of both the fraction and the absolute number of resistant cases.

Further studies by Hensley and colleagues (2011) reported antibody-driven antigenic variation in one domain of the H1 hemagglutinin segment sialic acid (Sa) site lead to compensatory mutations in the neuraminidase segment, resulting in antigenic variation and the development of drug resistance. This implies antiviral resistance may also rise from antibody driven hemagglutinin escape. Additionally, a review by Renaud, Kuypers and Englund (2011) emphasized the potential consequences of antiviral resistance in immunocompromised populations, where infected individuals experience increased risks of complications and transmissibility with limited therapeutic options.

Looking specifically in the tropical arena, research performed by Hurt and colleagues (2011) studied the development of resistance in the Asia-Pacific region between March 2009 and March 2010 by analyzing over 1400 isolates of 2009 pandemic H1N1 viruses for their susceptibility to neuraminidase inhibitors. They found an overall frequency of resistance of 1.1% (16 cases/1488 isolates tested). All sixteen viruses were classified as resistant to oseltamivir, and none were zanamivir resistant. Of the 16 detected mutants, nine were associated with current oseltamivir treatment, but the
remaining four mutants indicate a possibly limited, low-level community transmission pattern of oseltamivir resistant strains (Hurt et al., 2011).

An investigation by Miller et al. (2012) focused on searching for oseltamivir resistance in Hawai‘i and the United States Affiliated Pacific Islands, because of the considerable travel exposure. In their study, 263 pandemic H1N1 positive specimens were tested by pyrosequencing for the H275Y mutation conferring oseltamivir resistance, and all were found negative for the mutation. Researchers suggest contributing factors including geographic isolation, aggressive vaccination campaigns, judicious antiviral usage, the lack of a “second wave” of pandemic influenza, and below average tourism following the global economic crisis may have limited antiviral resistance, but counseled continued surveillance and vigilance is necessary (Miller et al., 2012).

**Meeting the Challenges of Influenza Control**

Early prevention of the spread of influenza viruses amongst humans and other animals has been shown as critical to limiting evolutionary reassortment thereby curbing selection for epidemic strains that possess more efficient transmission, immune evasion and drug resistance. The globalization of our economies necessitates geographically broad surveillance across multiple, linked regions. The complex nature of influenza replication requires multi-gene molecular surveillance in order to detect recombinants and resolve the evolutionary history of the viruses. The importance of swine and avian hosts in the evolution of influenza necessitates taxonomically broad surveillance, since the mixing of new genetic elements in swine typically has resulted in the emergence of viruses with human pandemic potential (Smith et al., 2009a). Furthermore, influenza in swine shows increased antigenic drift associated with reassortment, conferring even greater potential for evolutionary innovation and emergence (Vijaykrishna et al., 2011).

All three pandemics of the twentieth century, (1918 H1N1, 1957 H2N2 and 1968 H3N2 viral pandemics) were generated by a series of multiple reassortment events (antigenic shift) in swine or humans, and seem to have emerged over a period of years prior to gaining pandemic potential and recognition (Smith et al., 2009b). A global cooperation strategy for surveillance, as well as the control of pandemics through social restrictions,
vaccines, and antivirals is essential, especially where resource-rich countries share the latter with resource-constrained or resource-poor countries (Coburn et al., 2009).

Following surveillance measures, vaccination campaigns and the promotion of personal protection measures, such as hand washing and social distancing, designed to prevent disease susceptibility, initial infection and the spread of influenza. These need to be promoted as the individual’s first line of defense and are a critical component of influenza-related public health measures. However, vaccine efficacy is dependent on the ability of an integrated surveillance system to effectively predict future dominant circulating influenza strains in the Northern and Southern hemispheres. In terms of treatment of infected individuals, antiviral medications are available both as M2-protein inhibitors or neuraminidase inhibitors, but M2-protein inhibitors are no longer recommended and concerns about the rise of drug-resistant influenza strains makes usage less attractive for prophylactic purposes (Regoes & Bonhoeffer, 2006).

For population level influenza protection, a previous randomized trial suggested vaccination of school-age children and adolescents may significantly protect the larger community from influenza (Loeb et al., 2010). Vaccination of children may be additionally important since the annual attack rate of influenza among children appears highest among all age groups, with an estimated 10% - 40% infected each epidemic season, and children with underlying medical conditions bear a disproportionate burden of related morbidity and mortality (Effler, 2012; Bansal et al., 2010). Effler (2012) goes on to stress additional studies are needed to more firmly establish the impact of current vaccine recommendations. Additionally, Bansal et al. (2010) also reported school-age children experience the highest influenza attack rates in primary naive populations, but the burden shifts to adults in the following season, with implications for public health resource allocations.

Summary and Conclusions

Influenza holds enormous potential for evolutionary innovation through rapid RNA evolution because it has a segmented genome with the propensity to reassort coupled with the ability to infect multiple host species. In 2009, a quadruple reassortment in swine produced a pandemic H1N1 influenza subtype originating in Mexico, which
rapidly swept across the globe prompting the World Health Organization to declare a pandemic in June 2009 (Bansal et al., 2010; Garten et al., 2009). Throughout the course of the next year over 208 countries were affected (Bansal et al., 2010). Although mortality rates were generally low, there was a particular vulnerability identified in the 18 to 65 year old age group, and in those with underlying medical conditions (Fowlkes et al., 2011). In the Pacific island nations/territories, the pandemic was first recognized in June 2009 in French Polynesia and influenza infections among the 19 Pacific island countries and territories peaked in August 2009. Although pandemic preparation and outbreak response was in place, facilitated by multiple international partners, there was a significant impact on the region in terms of morbidity and mortality, consistent with other indigenous and low-resource settings (Kool et al., 2013).

In terms of influenza transmission patterns, model-based research suggested international connectivity, and preferred destinations, particularly Asia and Australia, may have important implications for understanding intercontinental influenza spread (Viboud et al., 2012). More biologically complex models of influenza transmission dynamics could be developed to include evolutionary potential, encompassing influenza variants across multiple species, particularly bird, pig and human populations (Vijaykrishna et al., 2011). In the tropics, influenza transmission generally occurs year-round, but in some areas there are two annual epidemic peaks associated with local rainy seasons (Leo et al., 2009). Significant influenza impacts have been demonstrated by studies conducted in Thailand and Singapore; however, minimal research has been conducted to study specific transmission dynamics in the region (Simmerman et al., 2006; Chow et al., 2006; Leo et al., 2009). Research also suggests the tropical climate may play a significant role in suppression of influenza transmission due to relatively high humidity and temperature (Barreca et al., 2012; Shaman & Kohn, 2008; Lowen et al., 2007).

One of the largest challenges facing influenza control is antiviral resistance, due to a combination of evolution and strong selection from overuse of drug compounds. Antiviral treatment is available in the form of neuraminidase inhibitors or, to a lesser extent, M2 protein inhibitors, but growing resistance to these drugs is a cause for concern (Regoes & Bonhoeffer, 2006). In an investigation conducted in the Asia-Pacific region,
an overall frequency of neuraminidase inhibitor resistance was reported as 1.1% of all viruses collected during the study period, however, methodologies indicated research was conducted primarily in the South Pacific, and was not representative of all Pacific island nations. There were also findings indicative of absence or limited, low-level community transmission pattern of oseltamivir resistant strains (Hurt et al., 2011; Miller et al., 2012). Potential solutions include vaccination policies and campaigns, as well as the promotion of personal hygiene measures, such as hand washing and appropriate social distancing. Previous research also suggests vaccination of school age children in particular may have more widespread implications in terms of community influenza protection (Loeb et al., 2010).

The importance of sustained vigilance, as well as continued and globally expanded surveillance, in human populations as well as domestic swine and avian populations is needed to control influenza and the evolution of pandemic strains. Influenza transmission models need to incorporate the evolution, development and spread of new influenza variants. Further research is also recommended in the areas of vaccine development and the drivers of antiviral resistance. To ultimately resolve the origins and dynamics of influenza with pandemic potential, a globally comprehensive view of the role of the Pacific region as a bridge between Southeast Asia, the Americas, the tropics and the temperate regions in transmission is essential.
CHAPTER 2
Hawaii’s Role in Influenza Transmission Patterns

Abstract
Investigation into global influenza transmission patterns has been a topic of study for the past several years. Previous studies have reported antigenic origins of many influenza viral strains initially circulate in Southeast Asia, and then spread throughout the globe. This model specifically notes patterns of transmission through the Asian continent, and further to the Americas. However, the model does not comment on circulation of virus through the Pacific islands or the state of Hawai‘i, although Hawai‘i in particular often serves as an important crossroad between the Asian continent and the continental United States. This chapter explores the potential importance of the state of Hawai‘i in influenza transmission patterns. Based on travel information and peak influenza transmission periods reported by the Hawai‘i Department of Health, Hawai‘i could act as an important arena for the co-circulation of viruses from different points of origin (the United States and Asia), and specifically increase the potential for co-circulation, and potential recombination of different strains. I conclude by discussing the potential use of state surveillance information for research to further describe Pacific influenza transmission patterns.

Introduction
Global patterns of influenza transmission have been an area of interest, study, and clinical relevance, for many years. A recent study (Russell et. al. 2008) had reported antigenic origins of influenza virus, specifically H3N2, initially developed in Southeast Asia, then spread throughout the globe from there. This model further suggested viral transmission through the Asian continent, which further progresses to the Americas. However, circulation of virus through the Pacific islands or the state of Hawai‘i was not noted. Another publication (Rambaut et al., 2008) described a “source and sink” model, in which many new viral lineages are sourced from a persistent “source” reservoir, proposed to be based in the tropics, and exported to “sink” populations in the Northern and Southern hemispheres. Furthermore, continuous transmission from “source” populations allowed for natural selection and antigenic diversity to develop more
efficiently than in the “sink” populations alone, which are limited by seasonal bottlenecks. This model again referenced the importance of transmission dynamics in the tropics, which include Hawai‘i, but did not suggest any specific pattern.

Hawaii’s central location in the Pacific, close to the equator separating the hemispheres and longitudinally between North America and Asia, ensures the state is exposed to individuals harboring a wide variety of infectious diseases, including influenza, as a result of well-trafficked travel patterns. Although the state often serves as a major crossroads bridging the Eastern and Western worlds, no special attention has been given to influenza transmission dynamics in and through Hawai‘i. Minimal molecular epidemiology research has been conducted in the state, particularly in comparison to work performed in the continental United States. Additionally, a better understanding of disease in Hawai‘i may have strong translational applications in infectious disease disaster preparation and response, particularly in regards to potential influenza pandemics, which are phenomena of recent concern.

Laboratory work may also be translated beyond purely scientific information into applied data for more efficient and appropriate disaster prevention and response. Integration of state governmental organizations, including the Hawai‘i Department of Health (HDOH) State Laboratories Division (SLD) and Disease Outbreak Control Division (DOCD), as well as other medical labs and disaster response/prevention agencies could lead to more optimal translation of the scientific research into appropriate applications. For example, the HDOH SLD, in cooperation with community laboratories, developed a strategy to decentralize molecular influenza testing capacity and established a testing algorithm for routine and potential pandemic testing (Whelen et. al. 2011).

**Influenza in a Tropical Environment**

Influenza viruses have a substantial impact on countries in tropical regions, including Hawai‘i. Agricultural practices, particularly those involving close proximity between domesticated animals and their human owners, combined with a tropical climate, can play an important role in influenza transmission. Consequently, influenza viruses also have a large economic impact in these areas. Descriptions of influenza transmission patterns through the tropical region are minimal; although it has been
suggested by several investigators that this area may play a significant role in global transmission (Leo et al., 2009; Hampson et al., 1999; Russell et al., 2008).

The tropical climate may significantly suppress influenza transmission due to relatively high humidity and temperatures. A study performed in the United States by Barreca and colleagues (2012) found low absolute humidity and low temperatures were an observed determinant of increased influenza mortality, even after controlling for temperature. These findings were further supported by Shaman and Kohn (2008), as well as by a study performed by Lowen and colleagues (2007) using guinea pigs as model hosts. Although these studies suggest the warm, humid climate of the tropics may confer some degree of protection; no research has yet been performed in, or in replication of, this environment. Furthermore this does not explain why Hawai’i has year-round influenza activity. One possibility is that the warm climate is associated with agricultural practices that foster close proximity between human populations and domesticated animals.

Domesticated animals throughout Asia-Pacific and Southeast Asian countries are generally ubiquitous components of small household farms. In a systematic review, 80% of the total poultry in many developing countries was identified in backyard flocks, which provide a food source for the families and may also provide a source of income (Conan et al., 2012). Small household farms are often associated with humans and animals residing in close proximity. In a 2008 study performed in two rural villages in Bangladesh, 92% of households reported flocks of backyard chickens, and many families reported poultry scavenging for food among household living areas, as well as drinking and bathing in the same water used by humans for bathing and washing of utensils and clothing. One village further reported poultry living in bedrooms with humans in 85% of households (Sultana et al., 2012).

In July 2012, a Maui teacher who also raised swine at home contracted a H3N2 variant influenza A virus that was known to be circulating in pigs. This H3N2 variant virus was only rarely associated with human cases and thus far human-to-human transmission has been inefficient. Influenza A (H3N2v) had only been reported in seven states other than Hawai’i, and 23/29 cases were associated with close direct contact
between humans and domestic swine, often at agricultural fairs (Centers for Disease Control and Prevention [CDC] 2012).

The importance of influenza surveillance in both human and animal populations, as well as integration of the two was discussed by Levings (2011) as a way to promote efficient disease preparation and response in the United States. The importance for more holistic surveillance was reinforced by a study of surveillance systems in Egypt, where agricultural practices are similar to those of Pacific and Southeast Asian regions. In the Egyptian study, a multivariate risk model correlated low temperatures, rural environments, high poultry density, and recent avian outbreaks with an increased risk of highly pathogenic avian influenza H5N1 cases in humans (Rabinowitz et al. 2012). Human cases of H5N1 were also temporally associated with outbreaks in poultry populations.

Agricultural practices associated with close proximity of humans and animals and the tropical climate may have a conflicting, but substantial impact on transmission of influenza viruses throughout the Southeast Asia and Asia Pacific regions and beyond. In turn, influenza viruses also have a significant economic impact in these areas. Investigators in Thailand have reported a productivity loss of between US$ 23.4 and US$ 62.9 million due to influenza virus (Simmerman et al., 2006). Also, studies in Singapore have found that a certain mutation (His275Y) conferring oseltamivir resistance was detected in approximately 80% of seasonal H1N1 influenza present in humans there in 2008 (Leo et al., 2009). In a separate study conducted between April 27 and May 24, 2009, research in Singapore indicated 24% of travelers with respiratory symptoms from swine-origin, 2009 pandemic H1N1 affected countries were infected with H3N2, 1.6% had seasonal H1N1 and 2.7% were positive for influenza B. Throughout tropical countries there is also minimal public health policy on vaccination, and a concerning presence of oseltamivir resistance to seasonal H1N1, which may potentially undermine pandemic preparation efforts, focused on antiviral stockpiling (Leo et al. 2009).

**Historical Relevance of Hawai‘i**

At a time when the globe was working towards recovery from the effects and aftermath of the First World War, the 1918 Spanish Influenza caused the most
devastating pandemic of the twentieth century, with a mortality rate eclipsing that of both world wars. The sickness was first diagnosed in the spring of 1918 and by the fall of 1919 approximately half of the total world population had been infected with the virulent influenza strain, resulting in the deaths of twenty to forty million individuals, including half a million Americans (Barry 2005).

The state of Hawai‘i experienced a relatively mild impact of the 1918 influenza pandemic, although historical documentation reports 1700 deaths between 1918 and 1920 resulting from the illness. Unlike most mainland areas, Hawai‘i experienced a higher incidence of influenza in 1919 and 1920, after the virus had done a great deal of damage elsewhere across the globe. There are accounts to verify the first wave of the virus arrived in July 1918 on ships arriving from both Chinese and Japanese ports. The first wave was short, lasting only from July to August of the same year, was seen only on the island of O‘ahu, and produced a relatively mild impact on the then U.S. territory. The second wave was significantly more severe and spread the virus to all islands between 1919 and into 1920, at which time, interestingly, high influenza mortality had nearly disappeared globally (Barry 2005; Morens & Taubenberger, 2009). The Hawai‘i Board of Health reported the number of cases exhibiting an infectious disease jumped to include approximately 12,000 more cases than the previous year, primarily due to the influx of influenza. In response, local hospitals experienced overcrowding, and the Queen’s Medical Center in downtown Honolulu was forced to set up tents on the front lawn in order to accommodate the growing sick population (Barry 2005). The delay in peak activity for the 1918 strain in Hawai‘i could be due to the fact that Honolulu was then a relatively small city, and travel to the state took days or weeks rather than hours.

Aside from the medical effects, the 1918 Spanish Influenza also had a significant impact in an economic sense, including productivity losses, high medical costs and increases in government expenditures to care for both affected, and unaffected, populations. In order to better prevent and prepare for influenza pandemics and epidemics it is important to develop an understanding of both the origins and transmission sequences of influenza A circulating in Hawai‘i.
Current Role of the State of Hawai‘i

The state of Hawai‘i is unique in many respects that warrant careful consideration with respect to the precipitation of an outbreak. There is a high movement of people, including tourists and military personnel, who represent potential carriers of infectious disease in and out of the state. Hawai‘i also sees a high number of travelers from Asia. According to recently published reports, Asian countries, particularly in the southeastern region, have been identified as potential origins for seasonal H3N2 influenza activity (Russell et al., 2008). It is also suggested that careful surveillance for influenza A in Southeast Asia may lead to a more accurate forecast of viral strains poised for circulation throughout other parts of the world. Hawai‘i serves as a crossroads between Southeast Asia and the continental United States, and is suspected to play an important role in the movement of influenza (Russell et al., 2008). These activities also have inherent seasonality: for example, travelers from Canada are more frequent between December and March (Hawai‘i Department of Business, Economic Development and Tourism [HDBEDT] “Annual Visitors By Month” 2012), and influenza transmission in the source populations is also highly seasonal, although with different peak times in the tropics versus the temperate zone, thus differentially affecting transmission to, and in, Hawai‘i. Additionally, there is a relatively high frequency of Hawai‘i residents traveling out of state during the winter holidays in December and January, which interestingly also correlates with Hawaii’s peak influenza activity in January.

As a major tourist destination, Hawai‘i has visitors from many different parts of the world. Four major regions, the United States (U.S.) West, the U.S. East, Japan and Canada, have consistently been the most important contributors to tourism, accounting for approximately of 88% of total annual visitor days from 2008-2011 (Table 1.1). Annual data for 2012 was not available the time of writing. Each of these four areas also has varying travel patterns. The U.S. West was the highest contributor of visitor days year round, and the highest number tourist numbers were in June, July, August and December. This pattern was present in 2011, 2010 and 2009, but in 2008 there was a slight variation as the highest visitor months from the U.S. West were seen in March, June, July and August. The U.S. East consistently contributes the second highest number of visitors each month throughout the year, with the highest tourist numbers in January,
June and July. February, March and December comprised a second tier. This pattern was replicated in 2011, 2010 and 2009, but varied slightly in 2008, when January, February, March, June and July were the high months (HDBEDT 2011, 2010, 2009, 2008).

Tourists from Japan exhibited more seasonally variable travel patterns. Highest visitor days were seen in July, August, September and December with a second tier observed in January, February and October. This pattern was replicated in 2011 and 2010, but in 2009 highest tier months included March, August, September and December, and in 2008 first tier months were March, July, August and December. Japanese contributions of monthly visitor days had more of a variation throughout the year compared to visitors from the U.S. mainland. Japanese tourism ranged from a high in August, 896,882 visitor days in 2011, to a low in April, 421,899 visitor days in 2011. The fourth group, Canadian visitors, exhibited the clearest travel pattern. Visitor days from Canada were highest in December, January, February and March, with April and November as clear transitional months. Monthly Canadian visitor days varied from a high in December, 882,466 visitor days in 2011, to a low in June, 182,582 visitor days in 2011 (HDBEDT 2011, 2010, 2009, 2008).

Table 1.1 Annual Visitor Days per Year

<table>
<thead>
<tr>
<th>Year</th>
<th>Total Visitor Days per Year</th>
<th>U.S. West* (%)</th>
<th>U.S. East* (%)</th>
<th>Japan* (%)</th>
<th>Canada* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>67,825,871</td>
<td>42.42</td>
<td>25.33</td>
<td>11.04</td>
<td>8.91</td>
</tr>
<tr>
<td>2010</td>
<td>64,951,433</td>
<td>43.06</td>
<td>25.89</td>
<td>11.20</td>
<td>7.92</td>
</tr>
<tr>
<td>2009</td>
<td>60,255,061</td>
<td>43.20</td>
<td>27.00</td>
<td>11.30</td>
<td>7.30</td>
</tr>
<tr>
<td>2008</td>
<td>63,130,133</td>
<td>42.21</td>
<td>27.86</td>
<td>10.68</td>
<td>7.34</td>
</tr>
<tr>
<td>2008-2011 Average</td>
<td>64,040,625</td>
<td>42.72</td>
<td>26.52</td>
<td>11.06</td>
<td>7.87</td>
</tr>
</tbody>
</table>

* Regional percentages are represented as a total annual percentage of total visitor days contributed by visitors from this area.

In regards to travelers from Japan specifically, research performed by Nishura and Inaba (2011) used data from influenza positive Japanese travelers who recently returned from Hawai‘i to model the daily frequency of illness onset after departure from the state.
Their study reported likelihood estimates of the median incubation period of the 2009 pandemic H1N1 virus range from 1.43 to 1.64 days, in accordance with varying modeling assumptions. These estimates were consistent with a previous study stemming from a New York school outbreak (Lessler, Reich & Cummings, 2009), and with previous virological estimates (Horsfall 1954). Such a short incubation period further highlighted the potential impact of infected travelers, which has particular importance in Hawai‘i.

Variables that affect influenza in source populations also affect behavior of the virus in geographically distinct recipient populations. This is particularly true when visitors originate from temperate regions. Influenza virus in the northern hemisphere temperate regions typically starts as early as October through April or May with a peak in transmission around January, although there is some variation from year to year (World Health Organization [WHO] 2012a). In the southern hemisphere temperate regions, an influenza season generally runs from April or May through the end of October, with a few specific countries, particularly in South America reporting slight variations in both initiation and completion of influenza transmission. In Australia and New Zealand, active influenza transmission begins in early June, peaks in mid-July and circulates through August with some slight yearly variations (WHO 2012b).

Influenza surveillance in Hawai‘i has indicated multiple patterns throughout the past several years. As of January 2013, in the current 2012–2013 influenza season, influenza activity in Hawai‘i has been dominated by H3N2 serotype. Since the beginning of the season in October, 9199 specimens had tested for influenza, 7277 (79.1%) by rapid antigen only and 1922 (20.9%) by more advanced methods. Of all specimens, 922 (10.0%) tested positive for influenza type A, and 151 (1.6%) tested positive for influenza type B. Of the 922 influenza A positives, 36 (3.9%) were positive for 2009 pandemic H1N1, none were positive for seasonal H1N1, 463 (50.2%) were positive for H3, and 423 (45.9%) were of a subtype yet to be determined. Positive cases reported by age group show individuals age 5-24 are most likely to test positive for influenza when suspected, accounting for 27% of all positive cases, although they contribute only 18% of specimens (HDOH 2012).

During the 2011-2012 influenza season in Hawai‘i, H3N2 also dominated, especially late in the season. Over the course of that influenza season, 9229 specimens
were tested, 6697 (72.6%) using rapid antigen only and 2532 (27.4%) by other methods. Of all specimens, 1273 tested positive for influenza A and 350 tested positive for influenza B. Of the influenza A positive isolates, 44 were identified as 2009 pandemic H1N1, and 1229 were H3 positive (A.C. Whelen, personal communication, April 3, 2013). Positive cases by age group were again dominated by age 5-24, as they accounted for 39% of all positive influenza cases although they contributed approximately 24% of the total specimens tested (HDOH 2013).

Influenza circulation was dominated by both 2009 pandemic H1N1 and H3N2 during the Hawai‘i 2010-2011 influenza season. Reports indicate 17,394 specimens were tested, with 5260 (30.2%) tested by methods other than rapid antigen. Of all specimens 867 were positive for influenza A and 264 were influenza B positive. Of influenza A positive isolates, 451 were 2009 pandemic H1N1, 1 was seasonal H1N1, and 416 were H3 positive (A.C. Whelen, personal communication, April 3, 2013). Of all specimens tested positive for influenza, age group 5-24 dominated with 49% of all positive tests, although this groups accounted for only 30% of specimens tested (HDOH 2011).

Throughout the 2009-2010 influenza season 2586 specimens were tested. Among them, 455 were positive for influenza A and 14 were influenza B positive. Of the influenza A positives, 439 were positive for 2009 pandemic H1N1, 15 were H3 positive, and 1 was seasonal H1N1 (A.C. Whelen, personal communication, April 3, 2013). Throughout the season, individuals age 5-24 comprised 35% of all positive influenza isolates, although they contributed only 20% of total specimens for testing. Within this season the WHO also declared the globe had moved from active pandemic into a post-pandemic period following the 2009 H1N1 pandemic (HDOH 2010).

It was towards the end of the 2008-2009 influenza season when the 2009 H1N1 pandemic strain was introduced to the state of Hawai‘i in May 2009. During the regular influenza season, with the additional out-of-season pandemic introduction, 45,948 specimens were tested. Among those, 9196 (20%) were identified as influenza A positive and 1200 (2.6%) tested positive for influenza B. Out-of-season, between May 10 and October 3, 2009 over 4900 influenza A positive specimens were subtyped. Of those, 2009 pandemic H1N1 was identified in 93% of all isolates and the remaining 7% were H3 positive. To provide a global context, at the same time the World Health Organization
had reported over 375,000 specimens of 2009 pandemic H1N1 had been laboratory confirmed, and there had been over 4500 pandemic-related deaths worldwide. Throughout the 2008-2009 influenza season in Hawai‘i, over 50% of all influenza positive isolates were collected from the age range of 5-24, although they contributed only 35% of all tested specimens (HDOH 2009).

The 2007-2008 influenza season was summarized as relatively mild in intensity. During this period, there were approximately equal proportions of seasonal H1 (dominant in fall 2007), H3 (dominant in winter 2007 through spring 2008) and influenza type B (dominant in summer 2008) co-circulated. H1N1 dominated the first half of the season, and continued to circulate through the end of the season although H3N2 and influenza type B activity began at low levels in January 2008 and continued throughout the reminder of the influenza season. In this time, 3571 specimens were tested, and of those, 1047 (29.3%) tested positive for influenza. Of the positives, 374 (36%) were of the H3 subtype, 334 (32%) were seasonal H1N1, 15 (1%) were not subtyped and 324 (31%) were positive for influenza type B. Again the age group of 5-24 year olds were the most likely to test positive when tested accounting for 32% of all influenza positive results even after contributing only 22% of specimens for testing (HDOH 2008).

Concentration of the state population also increases the risk of infectious disease. Approximately 75% of the state population resides in the city of Honolulu. The high population and small physical area force people into close proximity, with additional influence of individuals commuting in from sub-urban areas, encourage the rapid spread of infection. The urban living environment of Honolulu itself assists in rapid infection. The geographical isolation of the state, however, may prove beneficial in the case of a medical crisis, since Hawai‘i may be able to more closely monitor incoming and outgoing individuals.

**Hawai‘i Seasonal H1N1 Influenza Transmission Dynamics**

According to pilot study data of influenza viruses prior to 2009, there is phylogenetic evidence suggesting the state of Hawai‘i served as both an importer and exporter of seasonal H1N1 influenza virus, and may serve as an important crossroad between North American and Asian countries, as well as between hemispheres. Examples
of influenza strain importation have been found from the Philippines and Japan to Hawai‘i, subsequently followed by exportation to the continental U.S. (specifically Wisconsin, California, Minnesota and Washington). There are also examples of more international viral dynamics where Hawai‘i-based viral strains were interspersed with influenza from the Marshall Islands, Guam, China and Japan. Additionally, there are clades rich in Hawai‘i sequences interspersed with individual exportation events to the continental United States, suggesting interstate viral transmission prior to export. These patterns were derived from phylogenetic analysis of influenza strains identified by both geographic location as well as date to resolve temporal relationships between influenza strains.

Preliminary data suggests interesting transmission patterns are active, however, more data and further sampling are critical to developing a higher resolution picture of specific patterns through Hawai‘i. Associated location-data for Hawai‘i samples, if available, should also be used to describe transmission dynamics within the state.
CHAPTER 3
Molecular Epidemiology of Seasonal H1N1 Influenza in Hawai‘i

Abstract

Influenza virus poses significant human health challenges in the form of seasonal epidemics and pandemics in spite of vaccines and antivirals. Investigation of global influenza transmission patterns is of critical importance in controlling these outbreaks. The state of Hawai‘i is a diverse transmission arena influenced by intercontinental flyways to Asia, the Americas and Europe and often serves as an important crossroads between the Asian and North American continents. Periodic global travel is interspersed with extreme geographic isolation. Thus Hawai‘i could serve as an important melting pot for the co-circulation of viruses from other regions, contributing to the potential for reassortment of diverse viral strains. To determine the role of Hawai‘i in the transmission and evolution of influenza, I examined the temporal and spatial relationships of viruses isolated in Hawai‘i with respect to other isolates from around the world, including Asia, Oceania, the continental United States. I targeted seasonal H1N1 isolates collected between 2008 and 2009 from the viral archives of the Hawai‘i Department of Health, sequenced them for both the hemagglutinin and neuraminidase genome segments, and analyzed them in comparison to a selection of sequences from around the world. Phylogenetic analyses suggest Hawai‘i serves as both an importer and exporter of seasonal influenza - in some cases Hawai‘i isolates represent the earliest instance of a strain subsequently seen elsewhere; in other cases Hawai‘i isolates cluster with strains observed earlier in other areas. Significant differences were observed between the proportions of isolates imported to and exported from Hawai‘i for both hemagglutinin and neuraminidase analyses (chi-squared p<0.001). Variation across the hemagglutinin and neuraminidase genes over the period of study was insufficient to detect reassortment, and no recombination was detected within either gene. Selection analyses indicate several nucleotide sites under positive, diversifying selection for the hemagglutinin and neuraminidase. Together these results suggest that Hawai‘i is an important intermediary in the global circulation of seasonal influenza as both a source and sink population, and may be contributing to the selective environment.
Introduction

Seasonal influenza causes significant morbidity and mortality worldwide, with estimated deaths in the United States ranging from 3,000 to 49,000 per year between 1976 and 2007 (Thompson et al., 2010). Although vaccinations are available, they must be redesigned every season because influenza evolves such that previous vaccines are no longer effective. New strains evolve and emerge regionally and are rapidly redistributed on a global scale to pose new health challenges in the form of both seasonal epidemics as well as the occasional pandemic. An understanding of global influenza transmission patterns is critical for prevention, preparation and response to both seasonal epidemics and pandemics. Current literature suggests seasonal influenza, specifically H3N2, circulates and evolves in Southeast Asia before emerging from the region and spreading globally, initially through Asia then further to the Americas (Russell et al., 2008). A separate study (Rambaut et al., 2008) suggests a “source and sink” model where new viral lineages emerge from a persistent “source” reservoir, proposed to be based in the tropics, and are exported to “sink” populations in temperate regions of the Northern and Southern hemispheres. Additionally, influenza transmission from “source” environments allows for natural selection and antigenic diversity to evolve more efficiently than it would otherwise in “sink” populations alone where transmission is influenced by seasonal bottlenecks. The findings of both these studies suggest that Hawai‘i and the Pacific region could be important to global transmission patterns either as a gateway from Asia and the tropics to temperate regions, or as tropical source populations.

The state of Hawai‘i has a unique position, centrally located between Asia and the North American continent, which presents a diverse transmission arena influenced by well-trafficked global travel interspersed with geographic isolation. In many ways, Hawai‘i serves as an important crossroads between the eastern and western worlds; however, minimal molecular research concerning influenza has focused on this potentially critical area. A better understanding of influenza transmission dynamics through this region may have valuable implications for efficient and effective seasonal epidemic and pandemic preparation and response for the state of Hawai‘i, as well as for the continental United States, Oceania and Asian countries that serve as travel partners.
Travel information reports Hāwai‘i visitors originate from many different parts of the world, but predominantly four regions: the United States (U.S.) West, the U.S. East, Japan and Canada. These four areas have been found to account for an approximate average of 88.17% of total annual visitor days between 2008 and 2011. Specifically, and on average, between 2008 and 2011 the U.S. West has contributed 42.72%, U.S. East 26.52%, Japan 11.06% and Canada 7.87% of annual visitor days. Each of these areas is characterized by seasonal variations in influenza transmission, and in some cases, differ in peak periods for travel to Hāwai‘i. In general, December and January are when most people visit Hāwai‘i, during the winter holidays, and also when many Hāwai‘i residents travel out-of-state (DBEDT 2011, 2010, 2009, 2008). The December and January months also coincide with a time period when influenza virus in the northern hemisphere typically experiences peak transmission (WHO 2012a).

The purpose of this study was to describe the molecular epidemiology of H1N1 type A influenza virus in Hāwai‘i with reference to Asian, Oceania and North American countries. This study involved collaboration with Hāwai‘i Department of Health (HDOH) State Laboratories Division (SLD) and focused on confirmed cases of seasonal H1N1 influenza A in Hāwai‘i from 2008 and 2009. Seasonal H1N1 was identified as the most common seasonal strain in circulation over the time period 2007 to 2009. According to HDOH influenza surveillance reports produced by the Disease Outbreak and Control Division, the 2007 to 2008 influenza season saw relatively low levels of influenza-like illness reported. During the influenza season in Hāwai‘i, which intensifies from approximately October to May but continued at low levels year round, there were roughly equal proportions of H1, H3 and influenza type B circulating with H1 dominating the first half of the season, then continuing to circulate in a less dominant role thereafter. Comparatively, H3 and influenza type B activity began in low levels in January 2008 and continued throughout the remainder of the influenza season. During the 2008 to 2009 influenza season 20% of influenza-suspected samples collected tested positive for influenza A, compared to 2.6% positive for type B influenza. Of influenza A positive samples, 93% were subsequently positive for seasonal H1N1 strain, compared to 7% positive for H3N2. Beginning in May 2009 and thereafter, seasonal H1N1 was replaced in dominance by pandemic strain H1N1 (HDOH 2008; 2009; 2010).
To examine the role of Hawai‘i in the transmission and evolution of seasonal influenza, isolates were sequenced from 2008 – 2009, the earliest year samples were available (2008) and the last year before seasonal influenza was replaced by the pandemic strain (2009). This range includes multiple flu seasons, as “replicates” of influenza migration into and out of the state. The frequency of interannual sampling was designed to yield good resolution of within-season dynamics. Other studies have found significant variation in the hemagglutinin genome segment within a few transmission seasons to elucidate molecular epidemiologic patterns (e.g., Holmes et al., 2005).

Methods

Sample Selection

To best capture the evolutionary and epidemiologic dynamics of H1N1 within and between transmission seasons, I consulted the Hawai‘i State Department of Health (HDOH) Influenza Surveillance Reports published between 2008 and 2009, as well as the HDOH State Laboratories Division (SLD) database, and other health records. Random sub-sampling was partitioned across the entire period of transmission or season, proportionally intensified for periods of relatively high infection rates (Appendix A).

Comparison Sequence Selection

To determine the relationships of the Hawai‘i viruses to other isolates, it is critical to include a complete representation of potential associated strains. A comprehensive selection of influenza sequences for phylogenetic comparison was selected from the publically available and curated databases, with respect to location and time, National Center for Biotechnology Information (NCBI) Influenza Virus Resource database (Bao et al., 2008). Comparison sequences were selected using two processes. First, experimental sequences were cleaned, aligned and subjected to a pair-wise distance analysis (PAUP) to identify the two most divergent experimental isolates for each of the two alignments, hemagglutinin and neuraminidase segments. These divergent sequences were then processed through the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) and the 15 most similar sequences were downloaded for use as comparison data. The second selection process took advantage of the curated Influenza Virus Resource database to select full-length influenza A, human, H1N1 non-pandemic, non-identical
isolates from various localities in Asia, North American and Oceania collected between 2007 and December 31, 2009. The hemagglutinin search yielded 1035 sequences and the neuraminidase search yielded 970. Sequences were sorted by country/locality and date, and two were selected per country per available month, one early in the month (first listed, closest to the first of the month), and one from the middle of the month (first listed, closes to the 15th). Isolates from Pacific island and Southeast Asian countries were sampled more intensely due to their connections with Hawai‘i, and Southeast Asia’s importance in the influenza literature. Additionally, these areas generally report few isolates due to limited surveillance; as such, these regions were oversampled so as to normalize representation and minimize potential sampling bias. Isolates from Pacific island and Southeast Asian countries were sampled for up to five isolates per month. Japanese isolates were also sampled more intensely due to Japan’s travel connections with Hawai‘i, for up to four isolates per month. United States isolates were selected for two per region, except in the case of California, Oregon and Washington where two were selected per state per month when available, due to their relatively close geographic connection with Hawai‘i. All Hawai‘i sequences were selected for inclusion in analysis. A comprehensive list of sequences screened and included is available upon request.

**Sequencing and Analysis**

Viral RNA was extracted for selected H1N1 positive isolates from the HDOH state lab archives for Hawai‘i. RNA was reverse-transcribed into cDNA using Superscript III (Invitrogen), and each segment was then amplified using polymerase chain reaction (PCR) with a high-fidelity enzyme (PFU ultra II, Stratagene) and the appropriate primers (Appendix C). Successful amplification was confirmed by 1% agarose gel electrophoresis, and PCR product was purified by column (Qiagen). PCR products were sequenced using 11 (HA) and 9 (NA) unique primer reactions per amplicon designed for overlap coverage in both directions. Sequence reactions were sent for commercial sequencing to the Pacific Biosciences Research Center and the Advanced Studies of Genomics, Proteomics and Bioinformatics center, University of Hawai‘i. Derived sequences were trimmed, cleaned, and aligned using Sequencher v4.10 and Se-Al. Experimental sequences were aligned with NCBI sequences selected above using Clustal W. Alignments were verified in Se-Al.
To resolve the relationships between influenza strains from Hawai‘i, Asia, Oceania and North America, maximum-likelihood (ML) phylogenetic trees were generated for both hemagglutinin and neuraminidase alignments, using RaxML Blackbox (Stamatakis et al 2008). Analyses employed an evolutionary model as determined by Datamonkey 2010 evolutionary web server (Delport et al., 2010), namely the general-time-reversible substitution model with a gamma-shaped among-site heterogeneity parameter (GTR+Γ). For tree support, 100 ML bootstrapping replicates were implemented. Phylogenies of sequences labeled for country/locality and date of isolation were examined for clustering of Hawai‘i variants with other sequences based on geography and/or time. Summary tables were created based on the phylogenies to test whether Hawai‘i viruses more often originated from (were preceded by) a given location, implying importation, and/or was subsequently transmitted to (proceeded by) a given location, implying exportation. Patterns in which temporal order was unresolved suggest ongoing viral transmission in that areas involved and were excluded from the analysis. Statistical analyses implemented chi-squared tests using SAS v9.2 to investigate significant differences. Phylogenetic structure was compared for HA and NA to determine if incongruences between trees occurred, suggestive of reassortment. Phylogenetic trees were trimmed to coding data for selection analyses using HyPhy software. Both Single Breakpoint Recombination (SBP) and Genetic Algorithm for Recombination Detection (GARD) analyses were used to determine whether recombination had occurred within either hemagglutinin or neuraminidase segments. For selection analyses, two algorithms, Mixed Effects Model Evolution (MEME) and Fast Unbiased Bayesian Approximation (FUBAR), were performed to investigate positive selection among nucleotide sites in coding regions.

Results

To determine the evolutionary and migration dynamics of seasonal influenza A H1N1 viruses in Hawai‘i, archived virus isolates from the Hawai‘i State Department of Health were sequenced, resulting in 35 HA and 30 NA sequences of high quality quad-directional coverage. HA sequences from Hawai‘i were aligned with 398 publically available sequences representing 21 countries and spanning three years (2007-2009). NA
sequences from Hawai‘i were aligned with 375 reference sequences spanning 23 countries and the same three-year period. The percent variation across the alignment was 3.38% for HA and 2.75% for NA.

**Phylogenetic Analyses**

Phylogenetic analysis of influenza evolution, based on hemagglutinin and neuraminidase across two seasons (2007 to 2008 and 2008 to 2009), indicated that Hawai‘i sequences were integrated with sequences of global distribution segregated into several distinct lineages throughout the study period (Figures 3.1 and 3.2). Both the hemagglutinin and neuraminidase phylogenies are structured into two main lineages, one dominant in 2007 (lineage I, Figures 3.1 and 3.2), and one that contains some 2007 sequences and persisted until 2009 (lineage II, Figures 3.1 and 3.2). Thus lineage II appears to have gradually replaced lineage I. Both lineages are distributed globally and appear to have emerged from Asia based on the inclusion of some of the earliest sequences (e.g., China, 2006, in lineage I, and Japan, 2007, in lineage 2). No major exclusively Hawaiian lineages emerged, although some minor lineages did form over smaller time scales (within a month or a few months). This suggests that Hawai‘i is closely linked to other areas in terms of transmission although there might be short-term opportunities for Hawai‘i-exclusive transmission on an evolutionary scale.

There were no significant differences in phylogenetic structure at major nodes, (supported by bootstrap values over 75), to indicate biologically relevant reassortment. Recombination within each segment was also not detected according to both the Single Breakpoint Recombination (SBP) and Genetic Algorithm for Recombination Detection (GARD) tests implemented in Datamonkey.
Figure 3.1: Seasonal Hemagglutinin Segment Maximum Likelihood Phylogenetic Tree

Figure 3.1. Maximum likelihood (ML) phylogenetic tree based on the hemagglutinin with 100 ML bootstrap support shown for appropriate nodes. Sequence labels include NCBI Genbank accession number or internal sample code, location and date (month, day, year). Sequences from Hawai‘i are labeled in red, with those acquired in this study in red bold italics. Sequences from the western hemisphere (i.e., United States, Canada, Central America) are shown in blue. Sequences from the eastern hemisphere (Middle-east, Asia, Australia) are shown in black. Major lineages I and II are delineated on the right.
Figure 3.2: Seasonal Neuraminidase Segment Maximum Likelihood Phylogenetic Tree

Figure 3.2. Maximum likelihood (ML) phylogenetic tree based on the neuraminidase with 100 ML bootstrap support shown for appropriate nodes. Sequence labels include NCBI Genbank accession number or internal sample code, location and date (month, day, year). Sequences from Hawai‘i are labeled in red, with those acquired in this study in red bold italics. Sequences from the western hemisphere (i.e., United States, Canada, Central America) are shown in blue. Sequences from the eastern hemisphere (Middle-east, Asia, Australia) are shown in black. Major lineages I and II are delineated on the right.

Spatio-temporal Relationships

A systematic analysis of the geographic and temporal order of relatedness between Hawaiian sequences and global comparison sequences illustrated two distinct
patterns of influenza importation from different regions. During the 2007 to 2008 influenza season, Hawai‘i isolates primarily originated from Asia or the Asia-Pacific regions. This combined area, denoted Southeast Asia, Asia-Pacific and Other Asia, contributed 89.66% (26/29 isolates) of the sequences preceding viruses reported in Hawai‘i based on the hemagglutinin phylogeny. Similarly, 63.33% (19/30 isolates) of viruses preceding those isolated in Hawai‘i originated in Asia based on the neuraminidase.

Comparatively, virus originating in North America, including the continental United States (U.S.), Mexico and Central America, more prominently dominated the 2008-2009 influenza season in Hawai‘i. This region was responsible for 76.19% (32/42 isolates) of preceding hemagglutinin sequences among Hawai‘i viruses. Analyzed neuraminidase segments supported the trend as 81.82% (18/22 isolates) originated from North America. Interestingly, it may have been continuation of this North American pattern, which facilitated the spread of 2009 pandemic H1N1 influenza into Hawai‘i in April 2009. Furthermore, in an outbreak investigation accompanying the first Hawai‘i cases of pandemic influenza, 42 cases associated with previous U.S. mainland travel were confirmed prior to the first Hawai‘i-based cases on April 29th, 2009 (Park et al., 2009). A systematic breakdown of Hawai‘i sequences and their antecedents in time and phylogenetic structure by country is described in Table 3.1.
Similar analyses based on phylogenetic structure to determine those geographic regions likely to receive viruses from Hawai‘i indicated that Hawai‘i influenza sequences are most often proceeded by, or exported to, the continental United States, or they persist only in Hawai‘i, indicated by Hawai‘i isolates that were the latest temporal isolates in their respective clades. This pattern seems to be exhibited strongly during both the 2007 to 2008 influenza seasons, when 7 of 10 proceeding patterns of hemagglutinin
circulation, and 9 of 11 proceeding patterns of neuraminidase circulation, were reported to either progress to the continental U.S. or persist in the state of Hawai‘i, and during the 2008 to 2009 influenza season, when all 7 hemagglutinin proceed patterns and all 5 neuraminidase proceed patterns reflected either persistence in Hawai‘i or continuation to the continental U.S. A systematic review of these patterns is depicted in Table 3.2. The original phylogenetic summary table is provided in Table 3.3 and illustrates for each one, or a group of Hawai‘i sequences in a cluster, the preceding and proceeding sequences in time and their geographic association.

**Table 3.2: Phylogenetic Summary Table of Geographical Destinations of Seasonal Isolates Proceeding Hawai‘i Isolates**

<table>
<thead>
<tr>
<th>Influenza Season</th>
<th>Influenza Segment</th>
<th>Geographical Category</th>
<th>Number of Proceed Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007-2008</td>
<td>HA</td>
<td>Continental U.S.</td>
<td>4 (40%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>South-East Asia</td>
<td>1 (10%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Asia</td>
<td>2 (20%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Persist in Hawai‘i</td>
<td>3 (30%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Total HA Patterns</strong></td>
<td><strong>10</strong></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>Continental U.S.</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>South-East Asia</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Asia</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Persist in Hawai‘i</td>
<td>5 (45.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Total NA Patterns</strong></td>
<td><strong>11</strong></td>
</tr>
<tr>
<td>2008-2009</td>
<td>HA</td>
<td>Continental U.S.</td>
<td>5 (71.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>South-East Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Persist in Hawai‘i</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Total HA Patterns</strong></td>
<td><strong>7</strong></td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>Continental U.S.</td>
<td>2 (40%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>South-East Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Persist in Hawai‘i</td>
<td>3 (60%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Total NA Patterns</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>
Table 3.3: Phylogenetic Summary Table of Geographical Origins of Seasonal Isolates Preceding & Proceeding Hawai‘i Isolates

<table>
<thead>
<tr>
<th>Gene Segment</th>
<th>Hawai‘i Taxa Identifier</th>
<th>Hawai‘i Replicates in clad</th>
<th>Hawai‘i Data Range Start</th>
<th>Hawai‘i Data Range End</th>
<th>Precede</th>
<th>Precede Category</th>
<th>Proceed</th>
<th>Proceed Category</th>
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<tbody>
<tr>
<td>HA</td>
<td>EU811979</td>
<td>1</td>
<td>1/1/2008</td>
<td></td>
<td>Hawai‘i</td>
<td>Hawai‘i</td>
<td>Vietnam</td>
<td>SE Asia</td>
</tr>
<tr>
<td></td>
<td>HA0962592</td>
<td>1</td>
<td>3/17/2009</td>
<td></td>
<td>Minnesota</td>
<td>US-Mid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HA0609531</td>
<td>1</td>
<td>1/20/2009</td>
<td></td>
<td>Boston</td>
<td>US-East</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GO463567</td>
<td>1</td>
<td>10/20/2008</td>
<td></td>
<td>Nicaragua</td>
<td>Central Am</td>
<td>Utah</td>
<td>US-West</td>
</tr>
<tr>
<td></td>
<td>GO475840</td>
<td>14</td>
<td>10/18/2008</td>
<td>2/24/2009</td>
<td>Nicaragua</td>
<td>Central Am</td>
<td>Japan</td>
<td>Asia</td>
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<tr>
<td></td>
<td>HA096181</td>
<td>1</td>
<td>2/10/2009</td>
<td></td>
<td>Nicaragua</td>
<td>Central Am</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EU811981</td>
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<td>1/17/2008</td>
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<td>North Carolina</td>
<td>US-East</td>
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<tr>
<td></td>
<td>EU516082</td>
<td>7</td>
<td>10/1/2007</td>
<td>10/22/2007</td>
<td>Australia</td>
<td>Asia-Pacific</td>
<td>California</td>
<td>US-West</td>
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<tr>
<td></td>
<td>EU779620</td>
<td>1</td>
<td>1/25/2008</td>
<td></td>
<td>Australia</td>
<td>Asia-Pacific</td>
<td>Japan</td>
<td>Asia</td>
</tr>
<tr>
<td></td>
<td>HA082287</td>
<td>2</td>
<td>7/26/2008</td>
<td></td>
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<td>US-East</td>
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<td>EU567019</td>
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<td>1/14/2007</td>
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<td>Oregon</td>
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<td></td>
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<td>Asia-Pacific</td>
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<tr>
<td></td>
<td>EU516087</td>
<td>11</td>
<td>8/20/2007</td>
<td>2/21/2008</td>
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<td>2/21/2008</td>
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<td>Asia</td>
<td>Minnesota</td>
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<td>11/14/2007</td>
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<td>SE Asia</td>
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<td>US-West</td>
</tr>
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<td>N082287</td>
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<td>2/26/2008</td>
<td></td>
<td>Japan</td>
<td>Asia</td>
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<td>US-Mid</td>
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<td>N082259</td>
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<td>North America</td>
<td>Tennessee</td>
<td>US-East</td>
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<td>N082234</td>
<td>1</td>
<td>2/23/2008</td>
<td></td>
<td>Hong Kong</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>GO475758</td>
<td>1</td>
<td>12/17/2008</td>
<td></td>
<td>Thailand</td>
<td>SE Asia</td>
<td>Myanmar</td>
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<tr>
<td></td>
<td>NA090207</td>
<td>1</td>
<td>1/6/2009</td>
<td></td>
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<td>GO476079</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>EU811980</td>
<td>1</td>
<td>1/11/2008</td>
<td></td>
<td>Illinois</td>
<td>US-Mid</td>
<td>Japan</td>
<td>Asia</td>
</tr>
</tbody>
</table>

Statistical tests for significant patterns in Table 3 indicated that both preceding and proceeding sequence patterns for the hemagglutinin and neuraminidase segments were statistically independent and non-random for geographic category (chi-squared test, p<0.0001, for each of four analyses). Patterns in Hawai‘i preceding (implying importation) and proceeding (implying exportation) sequences for hemagglutinin were also significantly independent according to specific location (chi-squared test, p<0.0001 for both analyses). In specific location tests of precede and proceed patterns in neuraminidase the chi-squared test was restricted by a small sample size, rendering interpretation of the test outcomes inappropriate. Additionally, the frequency with which
hemagglutinin sequences in Hawai‘i were preceded by western United States sequences differed significantly, and preceding more often, from non-western United States sequences (p<0.0001). Similar analysis for neuraminidase segment was not statistically significant. For neuraminidase, Southeast Asia and Asia-Pacific origin influenza sequences were each statistically different from all other sequence categories in frequency they preceded Hawai‘i sequences (p<0.0001, two analyses). Similar analyses for neuraminidase segment were statistically significant comparing Southeast Asia to all other sequences (p<0.0001), and Southeast Asia to other Asian sequences (p=0.0004), but not significant in comparing Asia-Pacific preceding sequences from all others. Together these results indicate the influenza transmission patterns based on phylogenetic analysis are statistically distinct from random viral distributions and exhibit spatio-temporal structure.

Selection Analyses

To test for the influence of natural selection in the evolutionary history of influenza viruses over the study period, two different selection analyses were conducted for each hemagglutinin and neuraminidase influenza segments. Mixed Effects Model Evolution (MEME) and Fast Unbiased Bayesian Approximation (FUBAR) analyses were performed to investigate evidence of positive selection in the coding regions of either hemagglutinin or neuraminidase segments. Since they utilize different algorithms, results varied (Table 4). Significant evidence of positive selection, reported by both analyses, was identified at four sites (200, 202, 203 and 205) of the hemagglutinin segment, and none in neuraminidase.

Table 3.4: Analyses for Positive Selection Among Hawai‘i Influenza Isolates

<table>
<thead>
<tr>
<th>Genome Segment</th>
<th>Site/ Codon</th>
<th>Strength of Selection (ω) - FUBAR Analysis</th>
<th>Strength of Selection (ω) - MEME Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>169</td>
<td>4.015</td>
<td>X</td>
</tr>
<tr>
<td>HA</td>
<td>176</td>
<td>4.738</td>
<td>X</td>
</tr>
<tr>
<td>HA</td>
<td>200</td>
<td>4.423</td>
<td>Inf.</td>
</tr>
<tr>
<td>HA</td>
<td>202</td>
<td>4.921</td>
<td>Inf.</td>
</tr>
<tr>
<td>HA</td>
<td>203</td>
<td>9.388</td>
<td>9.421</td>
</tr>
<tr>
<td>HA</td>
<td>205</td>
<td>3.692</td>
<td>Inf.</td>
</tr>
<tr>
<td>NA</td>
<td>275</td>
<td>9.446</td>
<td>X</td>
</tr>
</tbody>
</table>
Discussion and Conclusions

A strong understanding of global influenza transmission has important implications for prevention, preparation and response to both seasonal epidemics and pandemics. Recent literature suggests Southeast Asia plays a critical role in influenza circulation prior to emergence and global spread (Russell et al., 2008). A separate study cites a source and sink model where viral lineages emerge from a persistent “source” reservoir based in the tropics then are exported to spark epidemics in temperate regions (Rambaut et al., 2008). Both studies imply a potentially important role for the state of Hawai‘i due to its close proximity to and travel relationship with Southeast Asia and other Asian countries, as well as its geographical positioning in the tropics; however, to date, little investigation has been performed in this area.

In this study, phylogenetic analyses of seasonal H1N1 influenza in Hawai‘i, compared with isolates from Asia, Oceania and North America, indicate Hawai‘i viral origins were predominantly based in Asia during the 2007 to 2008 influenza season. This pattern was demonstrated in analysis of the hemagglutinin segment, as well as in neuraminidase. In contrast, North American origins were found to be more dominant during the 2008 and 2009 Hawai‘i influenza season. Export of viral strains from Hawai‘i generally demonstrates strong bias for export to North America, specifically the continental United States. In this way, there is evidence Hawai‘i serves as a significant importer and exporter of influenza, and does serve as a gateway between Asian and North American countries.

The division of the overall phylogenies of hemagglutinin and neuraminidase into two lineages, one that was dominant in 2007, and one that appeared to co-circulate and then replace it, persisting into 2009, suggests that there were fitness differences between these two lineages. Selection analyses over the period of the study support the importance of natural selection and pressure toward genetic variability, as opposed to genetic conservation, particularly in the hemagglutinin. This suggests that genetic variability, and associated immune escape, was an important factor in shaping the evolution of seasonal H1N1 from 2007 to 2009. Other studies report similar findings on the importance of natural selection for immune avoidance as a driver in the evolution of influenza (Bush et al., 1999; Bragstad et al., 2008).
This study demonstrates the importance of a global view in the evolutionary dynamics of influenza viruses. The variability of transmission dynamics between seasons calls for further investigation into potential explanations for these phenomena, including incorporation of more sophisticated and refined epidemiological data, spatio-temporal models and travel patterns. With an improved understanding of Hawaii’s role in global influenza transmission patterns, particularly as a sentinel state for influenza in the continental United States, surveillance focused in Hawai‘i could lead to more efficient and effective prevention, preparation and response to influenza outbreaks in North America.
CHAPTER 4
Molecular Epidemiology of 2009 Pandemic H1N1 Influenza in Hawai’i

Abstract
The world has experienced periodic outbreaks of pandemic influenza five times over the past century, the most recent being the 2009 H1N1 pandemic, which spread through more than 214 countries and reportedly caused over 18,209 deaths. This most recent pandemic outbreak is a sobering reminder of the importance of global surveillance efforts to determine where and when pandemic strains arise, and what are the evolutionary processes that generate them. An understanding of global influenza transmission dynamics will ultimately lead to more effective and efficient outbreak prevention, preparation, and response. Hawai’i represents a potentially significant crossroads between two major influenza transmission arenas: Asia and North America. Thus it presents an ideal location for surveillance, for new strains emerging from the tropics and/or arising from reassortment. To address the importance of Hawai’i in pandemic influenza global transmission and evolutionary patterns, I conducted a study in which 2009 pandemic H1N1 influenza viruses were systematically sampled from Hawai’i Department of Health viral archives of influenza collected between 2009 and 2011. Sequence data was analyzed using phylogenetic and statistical methodologies to determine the relatedness of influenza strains in Hawai’i relative to strains from other locations. Results based on the two major antigenic regions, hemagglutinin (HA) and neuraminidase (NA), demonstrate phylogenetic structure for the HA but minimal evolution in the NA over three years, spanning two influenza seasons (2009-2010 and 2010-2011) and the initial, out-of-season emergence of 2009 pandemic influenza. According to the HA results, Hawaiian viral lineages are most often preceded by strains from Asia, specifically Asia-Pacific and Southeast Asian regions. Chi-squared statistical analyses further support significant differences between isolates imported to and exported from Hawai’i for two separate analyses performed on the hemagglutinin segment (p<0.001 for both analyses). There was inadequate phylogenetic signal in the NA to determine whether reassortment had occurred post emergence, and no identified evidence of recombination within segments. There was, however, significant evidence of positive,
diversifying selection among nucleotide sites in the coding regions identified at one site (220) of the hemagglutinin segment, and one site (386) of the neuraminidase segment.

*Introduction*

The World Health Organization (WHO) declared a pandemic in June 2009 following emergence of a novel H1N1 influenza virus from Mexico in March of that year resulting from a quadruple reassortment event. Reassortment included viral lineages from North American and Eurasian swine, which provided a high level of infectivity to an otherwise poorly transmitting swine influenza strain (Garten et al., 2009). The recombinant strain quickly spread throughout the globe and by December of 2009 pandemic influenza had been reported in more than 208 countries (Bansal et al., 2010). Additionally, seasonal influenza vaccinations that year were proven ineffective at protecting against the new variant, requiring the development of a separate vaccine specifically for pandemic H1N1 over several months following the initial outbreak.

The pandemic H1N1 virus caused significant morbidity and mortality in the United States. U.S.-based data on laboratory-confirmed 2009 pandemic H1N1 collected between April 17 and July 23, 2009 report a total of 377 pandemic-associated deaths, and indicate a mortality rate of 0.12 deaths per 100,000 people, (95% confidence interval 0.08 – 0.16). Influenza activity varied by locality, but the second-highest mortality rate was identified in Hawai‘i (0.62 deaths per 100,000). Of the total pandemic-related deaths, 76% occurred in people age 18 to 65, which lies in sharp contrast to seasonal influenza where over 90% of influenza-related mortality occurs in individuals over 65. Additionally, the mortality rate of the 2009 pandemic influenza was generally lower compared to seasonal H1N1 influenza, and the majority of deaths (78%) were associated with underlying medical conditions (Fowlkes et al., 2011).

The first two community transmission cases of 2009 pandemic H1N1 influenza in Hawai‘i were reported on May 12, 2009, twenty-one days following the first Centers for Disease Control and Prevention (CDC) reported case. These initial cases were documented in an eight-grade student and a third-grade teacher at the same school. Prior to the school-based outbreak, 42 cases of pandemic H1N1 influenza had been confirmed, but all infections had been associated with mainland travel and were classified as
imported cases. From these two initial cases the virus quickly spread to multiple schools, and subsequently across the state (CDC, 2009). Between May 10th and October 3rd of 2009 the Hawai‘i Department of Health State Laboratories Division (SLD) had performed influenza subtyping on more than 4900 influenza A positive specimens, with a vast majority identified as novel, or pandemic, H1N1 (Hawai‘i State Department of Health, Disease Outbreak and Control Division 2009).

Hawai‘i is a unique environment as a subtropical locale with strong travel ties to Asia and the US, making it a potentially important locale for the investigation of influenza transmission patterns. The central geographical location of the Hawaiian Islands in the Pacific, between the North American and Asian continents, ensures the state is exposed to a wide range of infectious diseases, including influenza, as a result of well-trafficked travel patterns. In particular, Hawai‘i encounters many visitors from Asia, which is an area suggested by recent literature to play a critical role in both the emergence of seasonal influenza strains and global transmission patterns. Further, it has been suggested that more thorough surveillance of influenza A in Southeast Asia may lead to a more accurate forecast of viral strains poised for global emergence. It is also possible Hawai‘i, as an important crossroad between Asia and North America, may serve as a significant melting pot for the co-circulation of diverse viral strains to increase the potential for reassortment, and thus play an important role in influenza evolution and emergence (Russell et al., 2008).

The purpose of this study was to examine the temporal and spatial relationships of Hawai‘i pandemic H1N1 isolates and describe the molecular epidemiology with respect to other 2009 pandemic isolates from across the globe, specifically Asia, Oceania and North America. The project scope includes confirmed cases of pandemic H1N1 influenza in Hawai‘i collected between the onset of the pandemic in 2009 to 2011. During and after mid-year 2009 until 2012, influenza A prevalence in Hawai‘i was dominated by pandemic strain H1N1. The time frame of 2009 - 2011 was chosen so as to model the most current patterns of influenza transmission. The range also needed to be large enough to encompass multiple years, as “replicates” of influenza migration into the state, but tight enough to yield good resolution of within-year or seasonal dynamics.
Methods

Sample Selection

To determine how to best capture the evolutionary and epidemiologic dynamics of H1N1 within and between transmission seasons, I consulted the Hawai‘i State Department of Health (HDOH) Influenza Surveillance Reports published between 2009 and 2011, as well as the HDOH SLD database, and other health records. The breakdown of sample selection was made so as to distribute selection across the entire year, while taking a closer look at specific periods of relatively high infection rates and therefore larger virus population sizes.

There were two basic research questions to address: 1) Where does influenza in Hawai‘i come from? I addressed this question by sampling to represent a full year, to better capture early imports; and 2) when might one strain be replaced by another? I addressed this by sampling more intensely during peak transmissions to capture multiple co-circulating types, to examine the phylodynamics, or lineage replacement patterns. For sample selection, I employed stratified random sampling based on the Influenza Surveillance Reports published by the HDOH Disease Outbreak Control Division and influenza testing results available on the HDOH SLD database.

**Stratum #1: Temporal**: Based on normal seasonality of influenza in Hawai‘i, which, according to the MMWR spans week 40 (i.e. 10/9/2010) to week 39 of the following year (i.e. 10/1/2011).

**Stratum #2: Intensity**: This was categorized as “low,” “high,” and “intermediate”. “Low” and “high” are officially designated when the abundance of a week’s specimen number is greater than 2 standard deviations (that is, >95%) away from the mean of the previous 10 means. The “intermediate” category was identified 3 weeks prior to the beginning of the “high” intensity period, sampled to identify any pre-emergent viruses that spark “high” intensity transmission.

“Low” intensity sampling: 3 randomly selected samples every 3 weeks, (i.e. for the 2008-09 season, this encompassed 28 weeks for a total of 28 samples).

“High” intensity sampling: A minimum of 4 randomly selected samples per week, or 2% of the available weekly samples, whichever was greater, (i.e. for the 2008-09 season, this encompassed 16 weeks for a total of 64 samples).
“Intermediate” intensity sampling: 3 randomly selected samples per week. The defined 3 weeks prior to the “high” category gave rise to 9 samples total. The total sample number per year varied based on the distribution of “low” and “high” intensity throughout the year, but for the example of the 2008-2009 flu year I selected a total of 91 samples. Not all randomly selected samples were available in the freezer, so this number was ultimately reduced. Calculations relating to sample size are described in Appendix A, and a further breakdown of isolates selected is described in Appendix B.

Comparison Sequence Selection

A comprehensive selection of influenza sequences for phylogenetic comparison was selected from the publically available and curated, with respect to location and time, National Center for Biotechnology Information (NCBI) Influenza Virus Resource database (Bao et al., 2008). Comparison sequences were selected using two processes. First, experimental sequences were cleaned, aligned and subjected to a pairwise distance analysis (PAUP) to find the two most divergent experimental isolates for each hemagglutinin and neuraminidase segments. Sequence data corresponding to these two divergent isolates were run through the Basic Local Alignment Search Tool (BLAST) and the 15 most similar results were downloaded for use as comparison data. The second selection process involved searching through the influenza sequence database for nucleotide data corresponding to full-length influenza A, human, H1N1 pandemic only, non-identical isolates from Asia, North America and Oceania collected between 2009 and December 31, 2011. The hemagglutinin search yielded 3238 sequences and the neuraminidase search yielded 2225. Isolates from these two lists were sorted by country and date, and selection was performed separately for each hemagglutinin and neuraminidase. All Hawai‘i isolates were selected for inclusion in analysis, although few were found. In addition, two isolates were selected per country per available month, one isolate representing early in the month (the first listed isolate, as close to the first as possible in that month), and the other representing the middle of the month (the first listed isolate, as close to the 15th as possible). If less than three isolates were available per month, all available sequences were selected. Isolates from Pacific island and Southeast Asian countries were more intensely sampled and up to five isolates were selected per month when available, since these are areas of epidemiologic importance with strong ties
to Hawai‘i. Additionally, these areas generally report few isolates due to limited surveillance; as such, these regions were oversampled so as to normalize representation and minimize potential sampling bias. Japanese isolates were also intensely sampled, as an area with significant Asian-origin travel into Hawai‘i, and four isolates were selected per month when available. United States isolates were selected at the rate of approximately two isolates per region, except for California, Oregon and Washington, which were more intensely sampled due to their geographic connections with Hawai‘i, for up to two per state per month when available. A comprehensive list of included and excluded isolates is available upon request.

Sequencing and Analysis

Following sample selection from viral archives, viral RNA was extracted from isolates collected from individuals infected with H1N1 influenza in Hawai‘i. Infection and viral presence had been previously identified by the HDOH SLD. RNA was reverse-transcribed into cDNA using Superscript III (Invitrogen), and each segment was then amplified using polymerase chain reaction (PCR) with a high-fidelity enzyme (PFU ultra II, Stratagene) and the appropriate primers (Appendix C). Successful amplification was confirmed by 1% agarose gel electrophoresis, and PCR product was purified by column (Qiagen). Sequence reactions were sent for commercial sequencing to the Pacific Biosciences Research Center and the Advanced Studies of Genomics, Proteomics and Bioinformatics center, University of Hawai‘i. Sequencing reactions consisted of 11 unique primer reactions run on each amplicon, in both directions. The derived sequences were aligned using Sequencher v4.10 and Se-Al. Experimental sequences were aligned with NCBI sequences selected above using Clustal W. Alignments were verified in Se-Al. Based on the outcome of the hemagglutinin segment gel electrophoresis, a subset of successfully hemagglutinin-amplified isolates were amplified for the neuraminidase (NA) genomic segment, which was then amplified and sequenced as for HA.

To resolve the relationships between influenza strains from Hawai‘i, Asia, Oceania and North America, maximum-likelihood (ML) phylogenetic trees were generated for both hemagglutinin and neuraminidase alignments, using RaxML Blackbox (Stamatakis et al. 2008). Analyses employed an evolutionary model as determined by Datamonkey 2010 evolutionary web server (Delport et al., 2010), namely the general-
time-reversible substitution model with a gamma-shaped among-site heterogeneity parameter (GTR+Γ). For tree support, 100 ML bootstrapping replicates were implemented. Phylogenies of sequences labeled for country/locality and date of isolation were examined for clustering of Hawai‘i variants with other sequences based on geography and/or time. Summary tables were created based on the phylogenies to test whether Hawai‘i viruses more often originated from (preceded) a given location, and/or was subsequently transmitted (proceeded) to a given location. Statistical analyses implemented chi-squared tests to investigate significant differences. Phylogenetic trees were pruned of replicate comparison data and trimmed to coding data only for selection analyses using Datamonkey 2010 evolutionary web server (Delport et al., 2010). Phylogenies were compared for topological incongruences to determine the presence of reassortment. Recombination within either segment was investigated using Single Breakpoint Recombination (SBP) and Genetic Algorithm for Recombination Detection (GARD) analyses. To test for natural selection, Mixed Effects Model Evolution (MEME) and Fast Unbiased Bayesian Approximation (FUBAR) analyses were performed on the coding regions.

**Results**

Archived virus isolates from the Hawai‘i State Department of Health were sequenced, resulting in 134 viral sequences for the hemagglutinin segment and 25 for the neuraminidase segment of high quality four-fold sequence coverage in both directions. The hemagglutinin segment was aligned against 579 reference sequences spanning 33 countries and three years, spanning two influenza seasons (2009-2010 and 2010-2011) and the initial, out-of-season emergence of 2009 pandemic influenza. The neuraminidase segment was aligned against 491 reference sequences spanning 30 countries and the same time frame for a comprehensive phylogenetic analysis of the evolutionary dynamics of influenza through Hawai‘i and beyond. Percent similarity at the nucleotide level of aligned sequences from 2009-2011 ranged from 98.089% to 100% for hemagglutinin and 98.613% to 100% for neuraminidase.

During and following the 2009 H1N1 influenza pandemic Hawaii’s usual influenza season timeline was temporarily disrupted, although I did see periodic epidemic
viral peaks. As such, this study includes data corresponding to three years, spanning two influenza seasons (2009-2010 and 2010-2011) and the initial, out-of-season emergence of 2009 pandemic influenza.

Phylogenetic Analyses

Phylogenetic analysis of the hemagglutinin segment of pandemic H1N1 virus in Hawai‘i and around the world resolved into four main lineages over the study period (Figure 4.1). In most cases, Hawai‘i sequences were interspersed with strains from other locations, showing little local evolution. Lineages are as follows: lineage I includes the initial emerging lineage with sequences throughout initial, out-of-season pandemic emergence in mid-2009 and the beginning of the 2009-2010 season covering the entire breadth of geographic sources, reflecting its rapid global spread. From lineage I, emerged later, minor clusters of Hawaiian isolates, sometimes with U.S. isolates, stemming from Asian sequences (denoted Ia, b, c, and d) that circulated from the middle of the 2009-2010, and into 2010-2011 influenza seasons. However, the Hawaiian lineage dominant in the 2010-2011 influenza season fell into its own distinct cluster, denoted lineage II, that was exclusively Hawaiian and derived from Asian sequences.

Phylogenetic analysis of the neuraminidase segment of pandemic H1N1 virus in Hawai‘i over three years demonstrated substantial clustering of several Hawai‘i isolates together, exclusive of any other regions, into two main lineages (Figure 4.2). Lineage I was clearly the initial pandemic lineage, showing worldwide spread originating from the Americas. The Hawai‘i isolates within this lineage were unique and suggest some local evolution, possibly due to Hawai‘i’s relatively isolated geographic position. After the initial spread of lineage I beginning during the initial, out-of-season pandemic emergence in mid-2009, a second lineage formed in Asia, lineage II, which included another cluster of exclusively Hawaiian isolates. Sequences in lineage II were all post-2009. This pattern indicates Hawai‘i’s close connection to Asia, as well as its ability to serve as an arena for local evolution. Two isolated Hawai‘i sequences occurred separately amongst foreign sequences: one collected in July 2009 appeared to have been preceded both genetically and temporally, by a Chinese isolate collected in Guangdong in May 2009, suggesting viral importation; the second, collected on February 1, 2011 seemed to be preceded, genetically and temporally, by an Illinois isolate collected on January 26, 2011, again
implying importation. In general, phylogenetic analysis indicates the neuraminidase segment of the 2009 pandemic H1N1 virus has been highly conserved and has undergone minimal evolution over the past three years. Lack of evolution in the neuraminidase prevented formation and testing of summary tables of spatio-temporal relationships as for hemagglutinin (see below).

Figure 4.1: Pandemic Hemagglutinin Segment Maximum Likelihood Phylogenetic Tree

Figure 4.1. Maximum likelihood (ML) phylogenetic tree based on the hemagglutinin 134 sequences with 100 ML bootstrap support shown for appropriate nodes, rooted on the earliest pandemic strains from Mexico, April/May 2009. Sequence labels include NCBI Genbank accession number or internal sample code, location and date (month, day, year). Sequences from Hawaii are labeled in pink. Sequences from the western hemisphere (i.e., United States, Canada, Central America) are shown in blue. Sequences from the eastern hemisphere (Middle-east, Asia, Australia) are shown in black. Major lineages I and II are delineated on the right. Minor lineages containing Hawaiian strains (I a-d) are also indicated.
Figure 4.2: Pandemic Neuraminidase Segment Maximum Likelihood Phylogenetic Tree

Figure 4.2. Maximum likelihood (ML) phylogenetic tree based on the neuraminidase 25 sequences with 100 ML bootstrap support shown for appropriate nodes, rooted on the earliest pandemic strains from Mexico, April/May 2009. Sequence labels include NCBI Genbank accession number or internal sample code, location and date (month, day, year). Sequences from Hawai‘i are labeled in pink. Sequences from the western hemisphere (i.e., United States, Canada, Central America) are shown in blue. Sequences from the eastern hemisphere (Middle-east, Asia, Australia) are shown in black. Major lineages I and II are delineated on the right.

The difference in amount of phylogenetic structure between the hemagglutinin and the neuraminidase also rendered unreliable the comparison of topologies for incongruences consistent with reassortment. Tests for recombination within each segment, Single Breakpoint Recombination (SBP) and Genetic Algorithm for Recombination Detection (GARD) analyses, did not reveal any evidence of recombination within either sequence region.
Spatio-temporal Relationships

A systematic analysis of the spatio-temporal relationships between Hawaiian sequences and those from other regions based on the hemagglutinin demonstrated strong evidence of viral origins based in Asia over the study period; specifically the Asia-Pacific region during pandemic emergence in mid-2009 grouped at the origin of 83.3% (40/48 isolates) of total pandemic mid-2009 Hawai‘i isolates. Southeast Asia was subsequently the dominant observed contributor during the 2010-2011 influenza season, serving as the point of origin for 49.4% (39/79 isolates) of all 2010-2011 season Hawai‘i isolates. Overall, Asia-originating hemagglutinin segments preceded 100% (48/48 isolates) of Hawai‘i influenza during pandemic emergence in mid-2009, 75% (3/4 isolates) in the 2009-2010 influenza season and 79.74% (63/79 isolates) during the 2010-2011 season. Interestingly, this shift in contribution mirrors the HDOH Influenza Surveillance Reports, which describe a gradual decline in the dominance of pandemic H1N1 in the state beginning late in the 2009-2010 influenza season and continuing through 2010-2011, when pandemic H1N1 viral strains were reportedly co-dominant with H3N2 by the end of the identified 2011-2012 influenza season (HDOH 2012; HDOH 2013; HDOH 2011). To date, surveillance reports corresponding to the specified 2012 – 2013 influenza season are reporting dominance of the influenza A population by the H3N2 viral strain (HDOH 2012). A breakdown of geographic areas whose sequences precede Hawai‘i isolates is described in Table 4.1 below.
The phylogenetic relationships of Hawai‘i sequences to those that proceeded them, (implying exportation), based on the hemagglutinin data, indicates that Hawai‘i isolates were most likely to persist exclusively in Hawai‘i (i.e., Hawai‘i isolates were the latest temporal isolates in respective clades) throughout the entire study period: out-of-season pandemic emergence in mid-2009 (representing 3/7 “proceed” patterns), 2009-2010 influenza season (representing 2/4 “proceed” patterns) and 2010-2011 season (representing 7/9 “proceed” patterns), rather than proceed, or export, to another geographical area. However, during all three time periods there was some limited, identified transmission to both the Asia-Pacific and other Asian regions. A breakdown of these patterns is depicted in Table 4.2. The original phylogenetic summary table is provided in Table 4.3 and illustrates the geographic locations of sequences that precede
Hawai‘i sequences phylogenetically and in time, and the geographic locations of sequences that proceed Hawai‘i sequences phylogenetically and in time. Also included is the number of Hawai‘i isolates associated with each pattern for the hemagglutinin segment of the studied pandemic isolates.

**Table 4.2: Hemagglutinin Segment Phylogenetic Summary Table of Geographical Destinations of Pandemic Isolates Proceeding Hawai‘i Isolates**

<table>
<thead>
<tr>
<th>Influenza Season</th>
<th>Geographical Category</th>
<th>Number of Proceed Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Out of Season</strong></td>
<td>Continental U.S.</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td><strong>Pandemic 2009</strong></td>
<td>Asia-Pacific</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>3 (42.9%)</td>
</tr>
<tr>
<td></td>
<td>Total HA Patterns</td>
<td>7</td>
</tr>
<tr>
<td><strong>2009-10</strong></td>
<td>Continental U.S.</td>
<td>1 (25%)</td>
</tr>
<tr>
<td></td>
<td>Asia-Pacific</td>
<td>1 (25%)</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>2 (50%)</td>
</tr>
<tr>
<td></td>
<td>Total HA Patterns</td>
<td>4</td>
</tr>
<tr>
<td><strong>2010-11</strong></td>
<td>Continental U.S.</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td></td>
<td>Asia-Pacific</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>7 (77.8%)</td>
</tr>
<tr>
<td></td>
<td>Total HA Patterns</td>
<td>9</td>
</tr>
<tr>
<td>Influenza Season</td>
<td>Geographical Category</td>
<td>Number of Proceed Patterns</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td><strong>Out of Season Pandemic 2009</strong></td>
<td>Continental U.S.</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td></td>
<td>Asia-Pacific</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>2 (28.6%)</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>3 (42.9%)</td>
</tr>
<tr>
<td></td>
<td><strong>Total HA Patterns</strong></td>
<td><strong>7</strong></td>
</tr>
<tr>
<td><strong>2009-10</strong></td>
<td>Continental U.S.</td>
<td>1 (25%)</td>
</tr>
<tr>
<td></td>
<td>Asia-Pacific</td>
<td>1 (25%)</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>2 (50%)</td>
</tr>
<tr>
<td></td>
<td><strong>Total HA Patterns</strong></td>
<td><strong>4</strong></td>
</tr>
<tr>
<td><strong>2010-11</strong></td>
<td>Continental U.S.</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td></td>
<td>Asia-Pacific</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td></td>
<td>Other Asia</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Persist in Hawaii</td>
<td>7 (77.8%)</td>
</tr>
<tr>
<td></td>
<td><strong>Total HA Patterns</strong></td>
<td><strong>9</strong></td>
</tr>
</tbody>
</table>
Table 4.3: Hemagglutinin Segment Phylogenetic Summary Table of Geographical Origins of Pandemic Isolates Preceding & Proceeding Hawai‘i Isolates

<table>
<thead>
<tr>
<th>Hawai‘i Taxa Identifier</th>
<th>Hawai‘i Replicates in clade</th>
<th>Hawai‘i Data Range-Start</th>
<th>Hawai‘i Data Range-End</th>
<th>Precede</th>
<th>Precede Category</th>
<th>Proceed</th>
<th>Proceed Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA09p14804</td>
<td>3</td>
<td>7/6/2009</td>
<td>7/20/2009</td>
<td>Hong Kong</td>
<td>Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA09p131814</td>
<td>1</td>
<td>12/30/2009</td>
<td></td>
<td>Japan</td>
<td>Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA10p6277</td>
<td>8</td>
<td>9/14/2010</td>
<td>11/15/2010</td>
<td>Australia</td>
<td>Asia-Pacific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA10p6161</td>
<td>3</td>
<td>10/18/2010</td>
<td>11/9/2010</td>
<td>South Korea</td>
<td>Asia</td>
<td>Missouri</td>
<td>US- Mid</td>
</tr>
<tr>
<td>HA11p5085</td>
<td>5</td>
<td>3/9/2011</td>
<td>4/5/2011</td>
<td>South Korea</td>
<td>Asia</td>
<td>India</td>
<td></td>
</tr>
<tr>
<td>HA10p5812</td>
<td>1</td>
<td>10/18/2010</td>
<td></td>
<td>Singapore</td>
<td>Asia-Pacific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA11p0967</td>
<td>5</td>
<td>1/27/2011</td>
<td>4/11/2011</td>
<td>Thailand</td>
<td>South-East Asia</td>
<td>China</td>
<td>Asia</td>
</tr>
<tr>
<td>HA11p5160</td>
<td>1</td>
<td>4/5/2011</td>
<td></td>
<td>Singapore</td>
<td>Asia-Pacific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA09p19077</td>
<td>5</td>
<td>6/18/2009</td>
<td>1/11/2010</td>
<td>HI Origin</td>
<td>Hawai‘i</td>
<td>Malaysia</td>
<td>Asia-Pacific</td>
</tr>
<tr>
<td>HA09p26977</td>
<td>2</td>
<td>12/22/2009</td>
<td>1/12/2010</td>
<td>China</td>
<td>Asia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HA09p18182</td>
<td>4</td>
<td>6/22/2009</td>
<td>7/28/2009</td>
<td>Japan/Thailand</td>
<td>Asia/Q South-East</td>
<td>Asia</td>
<td></td>
</tr>
<tr>
<td>HA09p11206</td>
<td>1</td>
<td>6/25/2009</td>
<td></td>
<td>China</td>
<td>Asia</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Both preceding (implying importation) and proceeding (implying exportation) transmission patterns (Table 4.3) were shown to be statistically independent and non-random as organized by geographical category in two separate analyses (chi-squared, p<0.0001 for both). Two additional chi-squared analyses also supported independence and non-random patterns among specific preceding and proceeding locations (p<0.0001 for both). Chi-squared analyses were not performed for the neuraminidase segment, as a phylogenetic summary such as that organized in Table 4.3 would have been inappropriate for demonstrated patterns.

Selection Analyses

Two different selection analyses, Mixed Effects Model Evolution (MEME) and Fast Unbiased Bayesian Approximation (FUBAR) analyses, were performed to examine potential evidence of positive selection among nucleotide sites in the coding regions of
each segment. The methods employ different algorithms, and as such may produce different outcomes. Significant evidence of positive selection was considered if both tests reported it, and was identified at one site (220) of the hemagglutinin segment, and one site (386) of the neuraminidase segment (Table 4.4).

Table 4.4: Analyses for Positive Selection Among Pandemic Hawai‘i Influenza Isolates

<table>
<thead>
<tr>
<th>Genome Segment</th>
<th>Site/Codon</th>
<th>Strength of Selection (ω) FUBAR Analysis</th>
<th>Strength of Selection (ω) MEME Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>114</td>
<td>4.322</td>
<td>X</td>
</tr>
<tr>
<td>HA</td>
<td>220</td>
<td>5.355</td>
<td>Inf.</td>
</tr>
<tr>
<td>HA</td>
<td>338</td>
<td>X</td>
<td>Inf.</td>
</tr>
<tr>
<td>NA</td>
<td>35</td>
<td>X</td>
<td>Inf.</td>
</tr>
<tr>
<td>NA</td>
<td>386</td>
<td>10.257</td>
<td>Inf.</td>
</tr>
</tbody>
</table>

Discussion and Conclusions

In June 2009 the WHO officially declared an influenza pandemic following a quadruple reassortment event, which preceded viral spread through over 214 countries and reportedly caused over 18,209 deaths. The CDC first observed cases in the United States in April 2009, and on May 12th the first cases of autochthonous pandemic influenza transmission were confirmed in Hawai‘i. The state of Hawai‘i is a potentially important transmission and evolutionary environment for influenza, combining geographical isolation, unique positioning between Asian and North American continents and well-trafficked travel patterns. As such, an improved understanding of Hawaii’s role in global influenza transmission may have broad implications for improved effectiveness and efficiency in seasonal epidemic and pandemic prevention, preparation and response for Hawai‘i and beyond.

In this study, I sequenced 143 viruses for the hemagglutinin genome segment, and 36 for the neuraminidase segment for the purpose of examining the temporal and spatial relations of Hawai‘i pandemic H1N1 isolates, and to describe the molecular epidemiology of the state between mid-2009 and 2011 with respect to isolates from other
geographical areas, including Asia, Oceania and North American countries. Phylogenetic analyses suggested the hemagglutinin segment of the analyzed Hawai‘i isolates exhibited a strong pattern of origin in Asia, particularly the Asia-Pacific and Southeast Asian regions. Although most Hawai‘i transmission appeared to remain restricted to Hawai‘i, based on the geographic locations of those sequences proceeding from Hawai‘i circulation, there was also evidence of influenza export to both Asia and the continental United States. Chi-squared statistical analysis of hemagglutinin segment phylogenetic summary data further supported the independence and non-random nature of viral import to, and exports from, Hawai‘i on the levels of geographical category and specific geographical locale in four separate analyses (p<0.0001 for all four analyses).

Comparatively, the neuraminidase segment demonstrated minimal evolution and strong clustering of Hawai‘i isolates over the three years of the study period. No recombination was detected. Selection analyses performed on both hemagglutinin and neuraminidase segments found significant evidence of positive, diversifying selection in one nucleotide-coding site in hemagglutinin and in another coding site in neuraminidase.

This information supports the need for additional investigation into global influenza transmission patterns, and specifically on the role of Hawai‘i in these patterns. Research on the mechanisms behind the observed phylogenetic patterns may require the use of more refined epidemiological surveillance and travel pattern data. Investigational findings also suggest influenza viruses in Hawai‘i may be heavily influenced by earlier viruses originating in Asia, an area identified in the literature as significant in influenza transmission patterns (Russell et al., 2008). Data also suggests an implied importance for investigation of travel between North America and Asia, which may influence viruses subsequently seen in Hawai‘i, as during 2009 pandemic emergence it suggests the spread of the pandemic may have originated in Asia, thus confounding the actual origin-Mexico. An improved understanding of influenza viruses in and around the state of Hawai‘i, their transmission sources and sinks, as well as the evolutionary dynamics, may have important, broader implications for both North American and Asian nations.
CHAPTER 5
Conclusions and Implications

The purpose of this dissertation was to determine the role of Hawai‘i in the transmission and evolution of influenza, by examining the temporal and spatial relationships of viruses isolated in Hawai‘i with respect to other isolates from around the world, including Asia, Oceania, and the continental United States. To that end, I conducted a literature review focused on global influenza transmission patterns and evolutionary dynamics, a complex interaction between the tropics and temperate regions (Chapter #1), and specifically investigated the potential role of the state of Hawai‘i in influenza transmission patterns based on travel and epidemiology data (Chapter #2). To test these interactions, I systematically sampled seasonal H1N1 isolates collected between 2008 and 2009 (Chapter #3), and pandemic H1N1 isolates collected between 2009 and 2011 (Chapter #4), from the viral archives of the state of Hawai‘i Department of Health, sequenced them for both the hemagglutinin and neuraminidase genome segments, and analyzed them in comparison to a comprehensive selection of sequences from around the world.

Based on published studies, and supported by original research reported in this dissertation, it became evident that influenza transmission involved a complex interaction of environment, social practices, human movement and viral evolution. Contemporary literature has indicated the influence of climate, including temperature and humidity (Barreca et al., 2012; Shaman & Kohn, 2008; Lowen et al., 2008), the potential importance of viral surveillance in swine and avian populations (Smith et al., 2009a; Smith et al., 2009b), and the role of selective pressures on influenza viral mutations (Bragstad et al., 2008; Bush et al., 2009). Selection was a driving force for immune escape and vaccine ineffectiveness, and the increasing rate of resistance to oseltamivir and other antiviral medication, has been identified as an area of concern, especially in the Asia-Pacific region (Leo et al., 2009; Hurt et al., 2011).

Original research performed and reported as part of this dissertation also contributed to an improved understanding of Hawaii’s role in influenza transmission patterns. Phylogenetic analysis of both seasonal and pandemic influenza, hemagglutinin and neuraminidase segments, suggested Hawai‘i serves as both an importer and exporter
of influenza as well as a site for local evolution. Exportation of Hawaiian strains was observed when Hawai‘i isolates represented the earliest instance of a strain subsequently seen elsewhere; in other cases Hawai‘i isolates cluster with strains observed earlier in other areas, representing importation into Hawai‘i. General transmission patterns also suggested seasonal influenza in Hawai‘i during the 2007 to 2008 influenza season originated primarily from Asia (specifically Asia-Pacific for hemagglutinin and Asian continent for neuraminidase), and during the 2008 to 2009 season originated from both Central America and the continental United States (specifically for hemagglutinin more Central America, and for neuraminidase even between both regions). Additionally, seasonal influenza during the 2007 to 2008 season was more likely to proceed to the continental United States (hemagglutinin) or persist exclusively in Hawai‘i (neuraminidase). During the 2008 to 2009, Hawai‘i influenza more frequently proceeded to the continental United States (hemagglutinin) or persisted exclusively in Hawai‘i (neuraminidase).

Analysis of pandemic H1N1 influenza in Hawai‘i suggested hemagglutinin was preceded, (implying importation), more frequently by sequences from the Asia-Pacific region through 2009 and 2010, then more frequently by strains from Southeast Asia in 2011. Spatio-temporal viral patterns within and after Hawai‘i transmission indicated Hawai‘i influenza most frequently persisted exclusively in Hawai‘i from 2009 to 2011, however there were some isolated cases of viral export to the continental United States and the Asian continent. In summary, phylogenetic data from both seasonal and pandemic influenza provided evidence that Hawai‘i served as a significant importer and exporter of influenza and consequently served as a gateway between Asian and North American countries. There was no identified evidence of reassortment in any examined isolates, for either the hemagglutinin or neuraminidase segments during the study period (2007 to 2011). However, there was evidence of at least one significant site of positive selection identified for each seasonal and pandemic influenza in both hemagglutinin and neuraminidase segments (with the single exception of seasonal neuraminidase). Thus selection remains important even over the short periods of this study, and the presence of several uniquely Hawaiian lineages indicates local evolution.
This study supports the need for additional, continued investigation into global influenza transmission patterns, and specifically on the role of Hawai‘i in these patterns. Additionally, the variability of transmission between seasons suggests the need for further investigation into potential explanations for these patterns, including incorporation of more sophisticated and refined epidemiological data, spatio-temporal models and travel patterns. With an improved understanding of Hawaii’s role in global influenza transmission patterns, particularly as a sentinel state for influenza in the continental United States, surveillance focused in Hawai‘i could lead to more efficient and effective prevention, preparation and response to influenza outbreaks in North America.

Hawai‘i serves as an important transmission bridge between Asia, the Pacific, and the Americas, which justifies increased surveillance efforts, particularly as a proxy for areas with less intense surveillance and potentially high evolution, emergence, and transmission where emerging drug-resistance is present but difficult to monitor (Leo et al., 2009; Hurt et al., 2011), and in Southeast Asia, which is hypothesized to be a mixing region of influenza prior to global emergence (Russell et al., 2008). In light of logistical and economic challenges to influenza surveillance in the Pacific and Southeast Asian countries, Hawai‘i could serve as an important sentinel for distant landmasses while foreign surveillance systems are being developed. Until sustained surveillance systems are in place in the Pacific and Southeast Asia, it would be wise to continue aggressive influenza surveillance in Hawai‘i. Furthermore, surveillance in Hawai‘i may be more cost-efficient and feasible than areas in the continental United States, since Hawai‘i travel is limited to primarily airplane, with a minority by boat, and it is geographically constrained, making it hypothetically easier to implement a comprehensive surveillance program. In contrast, most metropolitan areas in the continental United States are larger with open boundaries, where travel to a place such as Los Angeles or New York may involve multiple airports, boats, trains, and cars.

The role of Hawai‘i as an important intermediary in the global transmission of influenza has been demonstrated. Future studies to further define this role and the mechanisms by which migration and local evolution occurs in Hawai‘i should include phylogenetic mapping of more specific Hawai‘i travel pattern data, into, out of, and
within the state; detailed phylogenetic studies of within-state transmission patterns; investigation of other epidemiological factors (e.g., vaccinations); and the inclusion of more Pacific island and Pacific rim samples for better resolution of influenza transmission patterns throughout the Pacific.
APPENDIX A
Seasonal Influenza Sampling Strategy and Sample Size Calculations

For seasonal H1N1 influenza random sample selection, because of the limited size of the isolate collection in freezer archives, ten randomly generated positions were generated for each available box of isolates. The isolate in the random position was identified, pulled and the testing results associated were checked using the Hawai‘i State Department of Health (HDOH) SLD database system. For identified positions that were found empty, a new random position number was generated. This strategy was applied to all available boxes of archived 2008 and 2009 influenza isolates (nine boxes for 2008 and ten boxes for 2009), therefore isolates were randomly selected across the entire available collection.

Assuming each event, or influenza strain, is independent and the frequency of a rare variant in the infected population is 10%, the following calculation was used.

Setting the p-value, the probability of not detecting a rare variant in the selected sample, to 0.001, \( P(x=0) = 0.001 = (1 - 10\%)^n \), where \( n \) is the minimum required sample size. Carrying the calculation out, \( n = \ln(0.001) / \ln(0.9) = 65.563 \). Therefore, using a sample size of 66 samples was adequate for a p-value of 0.001, (28.433 samples for a p-value of 0.05). Since there were 2 seasons of seasonal influenza being studied in the scope of the project, the total sample size should ideally include 132 seasonal isolates. To reach a p-value of 0.05, the total size must be no less than 57 seasonal isolates. This sample size was a balance between the need to paint a meaningful picture of influenza A activity in Hawai‘i and both cost and time of analysis.

Confirming the power of the test, when the sample size, \( n \), was set to 57 isolates for seasonal flu and the minimum proportion of viral variants to be detected was 95%, \( (1 - \alpha)^{57} = 0.05 \). Carrying out the calculation, \( \alpha = 0.0512 \approx 0.051 \). Therefore, if 57 isolates were sequenced, 95% of viral variants will be detected with a frequency of \( \alpha \approx 0.051 \).

This is a highly powerful design and could be modified somewhat to increase the number of seasons sampled back through time at a slightly lower power per year.

Sample Size Breakdown
Seasonal H1N1 2008
Total Samples Pulled: 108
Total Samples Seasonal H1N1 Positive: 13* (12.04% of total)

* Seasonal H1N1 2009
Total Samples Pulled: 131
Total Samples Seasonal H1N1 Positive: 50* (38.17% of total)

TOTAL Sample Size: Seasonal H1N1- 63 isolates

* Number sampled from collection may be less than the number of samples desired reflecting absences in the collection following lab-related removal (i.e. sample submission to the CDC for national surveillance purposes, sample use in other research studies or full sample volume used for routine testing). To address these gaps, each week was oversampled, (double the number of total samples desired were randomly selected and searched for in the freezer).
APPENDIX B
Seasonal Influenza Sampling and Lab Result Breakdown

**Hemagglutinin (HA) Segment**

*Note: PCR/Gel outcomes reflect results following re-amplification reactions if necessary. Sequence Data Clean-up Outcomes were categorized as “good” (clean, sequence of ≥ 1778 bp- whole HA segment), “mediocre” (contig with either gaps of varying sizes), or “failed” (individual sequence reactions unable to form a recognizable segment contig). Following discussion with Shannon Bennett, both good and mediocre contigs will be used in preliminary analysis to determine which mediocre contigs are of sufficient quality for analysis. Details regarding Sequence Data Clean-up procedures available upon request.

**Summary & Overall Totals**

Desired sample total: 57 seasonal H1N1 for p<0.05
Total sample number pulled from SLD freezer: 63 seasonal H1N1
Overall PCR/Gel Success: 45/63 = 71.4% seasonal H1N1
Overall Sequence Data Clean-up Success**: 31/45 = 68.9% seasonal H1N1

**Neuraminidase (NA) Segment**
*Note: PCR/Gel outcomes reflect results following re-amplification reactions if necessary. Sequence Data Clean-up Outcomes were categorized as “good” (clean, sequence of ≥ 1413 bp- whole NA segment), “mediocre” (contig with either gaps of varying sizes), or “failed” (individual sequence reactions unable to form a recognizable segment contig). Following discussion with Shannon Bennett, both good and mediocre contigs will be used in preliminary analysis to determine which mediocre contigs are of sufficient quality for analysis. Details regarding Sequence Data Clean-up procedures available upon request.

**Summary & Overall Totals**

Desired sample total: 20% of 188 HA positive samples = 38

Total tested: 99

Total PCR/Gel positive samples: 64 (31 seasonal + 68 pandemic)

Sequence Data Clean-up Outcomes: 40 good (25 seasonal + 15 pandemic), 12 mediocre (2 seasonal + 10 pandemic), & 10 failures (1 seasonal + 9 pandemic)

**Outcomes by Stratification**

**2008 Seasonal**

Sampled for Testing: 10

PCR/Gel Positive & Sequenced: 10

Sequence Data Clean-up Outcome: 10 good

**2009 Seasonal**

Sampled for Testing: 21

PCR/Gel Positive & Sequenced: 18

Sequence Data Clean-up Outcome: 15 good + 2 mediocre + 1 failed
APPENDIX C
Seasonal Influenza Primer Design

Amplification Primers

After a review of the existing literature, a PCR primer template was used from the sequence described in an article published in the Archives of Virology, “Universal primer set for the full-length amplification of all influenza A viruses” (Hoffmann et al, 2001). This sequence was found to be the most appropriate sequence match for the H1N1 strain being analyzed among those primer sequences described in the existing literature. The template was then compared to existing hemagglutinin (HA) gene sequences from 22 Hawai‘i H1N1 isolates available on GenBank. Alignments of the sequences were made using Sequencher and slight modifications were made to the primer sequences to better match Hawai‘i derived H1N1 influenza. The same process was applied to primer design for the neuraminidase (NA) gene segment.

Sequencing Primers

There were a total of 9 sequencing primers used for seasonal influenza viral analysis, including 4 forward and 5 reverse primers. These were all designed by the program PrimerSelect based on existing hemagglutinin (HA) gene sequences from 22 Hawai‘i H1N1 isolates available on GenBank. The sequencing primers were used in addition to the forward and reverse amplification primers in separate sequencing reactions to ensure full, bi-directional gene coverage.
APPENDIX D
Pandemic Influenza Sample Size Calculations

Assuming each event, or influenza strain, is independent and the frequency of a rare variant in the infected population is 10%, the following calculation was used.

Setting the p-value, the probability of not detecting a rare variant in the selected sample, to 0.001, \( P(x=0) = 0.001 = (1 - 0.10%)^n \), where \( n \) is the minimum required sample size. Carrying the calculation out, \( n = \frac{\ln(0.001)}{\ln(0.9)} = 65.563 \). Therefore, using a sample size of 66 samples was adequate for a p value of 0.001, (28.433 samples for a p-value of 0.05). Since there are 3 seasons of pandemic influenza being studied in the scope of the project, the total sample size should ideally include 198 pandemic isolates. To reach a p-value of 0.05, the total size must be no less than 85 pandemic isolates. This sample size was a balance between the need to paint a meaningful picture of influenza A activity in Hawai‘i and both cost and time of analysis.

Confirming the power of the test, when the sample size, \( n \), was set to 57 isolates for seasonal flu and the minimum proportion of viral variants to be detected was 95%, \( (1 - \alpha)^{57} = 0.05 \). Carrying out the calculation, \( \alpha = 0.0512 \approx 0.051 \). Therefore, if 57 isolates are sequenced, 95% of viral variants will be detected with a frequency of \( \alpha \approx 0.051 \).
When the sample size, \( n \), was set to 85 isolates for pandemic flu and the minimum proportion of viral variants to be detected was 95%, \( (1 - \alpha)^{85} = 0.05 \). Carrying out the calculation, \( \alpha = 0.03463 \approx 0.034 \). Therefore, if 85 isolates are sequenced, 95% of viral variants will be detected with a frequency of \( \alpha \approx 0.034 \). This is a highly powerful design and could be modified somewhat to increase the number of seasons sampled back through time at a slightly lower power per year.
### APPENDIX E

#### Pandemic Influenza Sample Selection Breakdown

**Pandemic H1N1 2009**

<table>
<thead>
<tr>
<th>Stratification</th>
<th>Total Strata</th>
<th>Sampling Strategy</th>
<th>Total Samples Desired</th>
<th>Number Samples from Collection</th>
<th>Percentage Pulled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>17</td>
<td>3/3 weeks</td>
<td>17</td>
<td>26</td>
<td>153%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>4</td>
<td>3/ week</td>
<td>12</td>
<td>9*</td>
<td>75%</td>
</tr>
<tr>
<td>High</td>
<td>14</td>
<td>4/ week or 2%</td>
<td>68</td>
<td>49*</td>
<td>72%</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td></td>
<td></td>
<td><strong>97</strong></td>
<td><strong>84</strong></td>
<td><strong>86.6%</strong></td>
</tr>
</tbody>
</table>

**Pandemic H1N1 2010**

<table>
<thead>
<tr>
<th>Stratification</th>
<th>Total Strata</th>
<th>Sampling Strategy</th>
<th>Total Samples Desired</th>
<th>Number Samples from Collection</th>
<th>Percentage Pulled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>23</td>
<td>3/3 weeks</td>
<td>23</td>
<td>21*</td>
<td>91%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>4</td>
<td>3/ week</td>
<td>12</td>
<td>0*</td>
<td>0%</td>
</tr>
<tr>
<td>High</td>
<td>4</td>
<td>4/ week or 2%</td>
<td>16</td>
<td>7*</td>
<td>44%</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td></td>
<td></td>
<td><strong>51</strong></td>
<td><strong>28</strong></td>
<td><strong>54.9%</strong></td>
</tr>
</tbody>
</table>

**Pandemic H1N1 2011**

<table>
<thead>
<tr>
<th>Stratification</th>
<th>Total Strata</th>
<th>Sampling Strategy</th>
<th>Total Samples Desired</th>
<th>Number Samples from Collection</th>
<th>Percentage Pulled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>3</td>
<td>3/3 weeks</td>
<td>3</td>
<td>8</td>
<td>267%</td>
</tr>
<tr>
<td>Intermediate</td>
<td>7</td>
<td>3/ week</td>
<td>21</td>
<td>43</td>
<td>205%</td>
</tr>
<tr>
<td>High</td>
<td>7</td>
<td>4/ week or 2%</td>
<td>28</td>
<td>44</td>
<td>157%</td>
</tr>
<tr>
<td><strong>TOTALS</strong></td>
<td></td>
<td></td>
<td><strong>52</strong></td>
<td><strong>95</strong></td>
<td><strong>183%</strong></td>
</tr>
</tbody>
</table>

**TOTAL Sample Size:** 207 pandemic isolates

* Number sampled from collection may be less than the number of samples desired reflecting absences in the collection following lab-related removal (i.e. sample submission to the CDC for national surveillance purposes, sample use in other research studies or full sample volume used for routine testing). To address these gaps, each week was oversampled, (double the number of total samples desired were randomly selected and searched for in the freezer), which accounts for occasional percentage pulled of >100%.
APPENDIX F
Pandemic Influenza Sampling and Lab Result Breakdown

Hemagglutinin (HA) Segment

*Note: PCR/Gel outcomes reflect results following re-amplification reactions if necessary. Sequence Data Clean-up Outcomes were categorized as “good” (clean, sequence of ≥ 1778 bp- whole HA segment), “mediocre” (contig with either gaps of varying sizes), or “failed” (individual sequence reactions unable to form a recognizable segment contig). Following discussion with Shannon Bennett, both good and mediocre contigs will be used in preliminary analysis to determine which mediocre contigs are of sufficient quality for analysis. Details regarding Sequence Data Clean-up procedures available upon request.

Summary & Overall Totals

Desired sample total: 85 pandemic isolates for p<0.05
Total sample number pulled from SLD freezer: 190 pandemic
Overall PCR/Gel Success: 143/190 = 75.3%
Overall Sequence Data Clean-up Success**: 108/143 = 75.5% pandemic

**Note: calculated as percentage total “good” sequences of total sent for sequencing

Pandemic Sample Outcome Totals

Pulled from SLD: 190
PCR/ Gel Outcomes: 143 positive + 1 faint positive + 24 negative + 22 consistent smear
Sent for Sequencing: 143
Sequence Data Clean-up Outcome: 108 good + 26 mediocre + 9 failed

Outcomes by Stratification

2009 Pandemic
Pulled from SLD: 70
PCR/ Gel Outcomes: 57 positive + 1 faint + 12 negative
Sent for Sequencing: 57
Sequence Data Clean-up Outcome: 43 good + 7 mediocre + 7 failed

2010 Pandemic
Pulled from SLD: 25
PCR/ Gel Outcomes: 20 positive + 5 negative
Sent for Sequencing: 20
Sequence Data Clean-up Outcome: 10 good + 10 mediocre

2011 Pandemic
Pulled from SLD: 95
PCR/ Gel Outcomes: 66 positive + 7 negative + 22 consistent, smeared gel
Sent for Sequencing: 66
Sequence Data Clean-up Outcome: 55 good + 9 mediocre + 2 failed

**Neuraminidase (NA) Segment**

*Note: PCR/Gel outcomes reflect results following re-amplification reactions if necessary. Sequence Data Clean-up Outcomes were categorized as “good” (clean, sequence of ≥ 1413 bp- whole NA segment), “mediocre” (contig with either gaps of varying sizes), or “failed” (individual sequence reactions unable to form a recognizable segment contig). Following discussion with Shannon Bennett, both good and mediocre contigs will be used in preliminary analysis to determine which mediocre contigs are of sufficient quality for analysis. Details regarding Sequence Data Clean-up procedures available upon request.*

**Summary & Overall Totals**

Desired sample total: 20% of 143 HA positive samples = 29
Total tested: 99
Total PCR/Gel positive samples: 36
Sequence Data Clean-up Outcomes: 15 good, 10 mediocre, & 11 failures

**Outcomes by Stratification**

**2009 Pandemic**
Sampled for Testing: 26
PCR/Gel Positive & Sequenced: 10
Sequence Data Clean-up Outcome: 3 good + 6 mediocre + 1 failed

**2010 Pandemic**
Sampled for Testing: 12
PCR/Gel Positive & Sequenced: 10
Sequence Data Clean-up Outcome: 2 good + 2 mediocre + 6 failed

**2011 Pandemic**
Sampled for Testing: 30
PCR/Gel Positive & Sequenced: 16
Sequence Data Clean-up Outcome: 10 good + 2 mediocre + 4 failed
APPENDIX G
Pandemic Influenza Primer Design

Amplification Primers

After a review of the existing literature, a PCR primer template was used from the sequence described in an article published in the Archives of Virology, “Universal primer set for the full-length amplification of all influenza A viruses” (Hoffmann et al., 2001). This sequence was found to be the most appropriate sequence match for the H1N1 strain being analyzed among those primer sequences described in the existing literature. The template was then compared to existing hemagglutinin (HA) gene sequences from 22 Hawai‘i H1N1 isolates available on GenBank. Alignments of the sequences were made using Sequencher and slight modifications were made to the primer sequences to better match Hawai‘i derived H1N1 influenza. The same process was applied to primer design for the neuraminidase viral segment. Although WHO published primers were tried, greater success was found by using the universal, segment-specific amplification primers.

Sequencing Primers

For pandemic influenza viral sequencing, primer sequences used by the World Health Organization (WHO) during the 2009 pandemic were published and made publicly available. The WHO primer sequences, (11 in total- 5 forward and 6 reverse), were used in addition to the universal, segment-specific, forward and reverse amplification primers in separate sequencing reactions to ensure full, bi-directional gene coverage.
LITERATURE CITED


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