AVIAN INFLUENZA VIRUS SURVEILLANCE IN CHILE AND COLOMBIA

By

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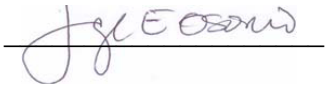
Avian influenza virus (AIV, Influenza A virus) is a pathogen of great public and animal health importance. Notably, an avian-like Influenza A virus was responsible for the death of millions of people during the "Spanish" 1918-1919 influenza pandemic. In Southeast Asia, highly pathogenic H5N1 influenza has continuously evolved and caused serious public health and economic concerns during the last two decades. Birds, mainly waterfowl and shorebirds, are the main reservoirs of Influenza A viruses in nature, therefore, significant effort has been invested in wild bird AIV surveillance. Surveillance efforts have been focused mostly in Asia, North America and Europe and have yielded a wealth of information about AIV ecology and phenotypic characteristics of viral isolates. However, AIV surveillance in other areas like Oceania, Africa and South America have been insufficient, hence the epidemiology of AIV in these regions is still poorly defined. Given the worldwide distribution of AIV and its transmission through wild birds, the purpose of this dissertation was to prove that a high diversity of AIVs, including potentially pathogenic subtypes, are currently circulating in many areas of South America. To this end, we engaged in active surveillance efforts in Chile and Colombia. Avian influenza surveillance had the double purpose of evaluating both the circulation of AIV in backyard poultry farms and wild birds. Backyard poultry farms are very common throughout South America and are known to provide an ideal interface for viruses of different origins, e.g., avian, swine and human, to intermix. Our results in Chile demonstrate that AIV is currently circulating in backyard poultry farms throughout our sampling area, as well as being present in several wild bird species. In wild birds, we obtained 16

AIV isolates, including subtypes of known pathogenic potential making this the richest collection of AIVs obtained in Chile to date. In Colombia, we were unable to obtain any viruses circulating in wild birds, however, for the first time in the continent, we described the circulation of AIV in a live animal market. These kinds of markets are a known source of interspecies transmission of AIV and have been credited with the emergence of several viral pathogens in the past. Even though viruses isolated from this market were not pathogenic in chicken and mice, they transmitted efficiently between poultry indicating the potential hazard these viruses pose to animals and humans. This research provides valuable information about the epidemiology of AIV in South America and indicates that further surveillance efforts throughout South America are needed in order to gain a thorough understanding of this pathogen in the region.

Approved by: 

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CHAPTER 1

INTRODUCTION

Influenza A

Influenza virus (IAV) is an enveloped and segmented negative-sense single-strand RNA virus with significant public health importance of the family *Orthomyxoviridae* genus Influenza A (1). Other members of the same genera are Influenza B and C and most recently, Influenza D (2,3). IAV has been isolated of several species, including a large variety of mammals and birds (1). Influenza is one of the most important respiratory pathogens in humans, causing up to 500,000 death annually (4). For the last 100 years, four identified pandemics and several other notable epidemics with millions of deaths worldwide have been caused by influenza A (5,6). Notably, an H1N1 avian-like Influenza A virus was responsible for the death of millions of people during the "Spanish" 1918-1919 Influenza pandemic (7). In recent years, an H5N1 avian origin influenza virus (AIV) has been responsible for hundreds of human deaths and millions of poultry fatalities since its dispersion from Southeast China in 1996 (8). Furthermore, a triple human, swine and avian reassortant virus caused the latest pandemic in 2009, that subsequently spread across the globe in less than a year despite several intergovernmental control efforts, resulting in an estimated loss of US $800 billion worldwide (6,9). Other current influenza subtypes of public health interest are H7N9 and H9N2 AIV strains, mainly circulating in China. While pathogenic H7N9 has causes 275 confirmed death since its first report in 2013, H9N2 is believed to circulate endemically in poultry populations of Africa, Asia and the Middle East, having so far caused only limited disease in humans (10).

Influenza A viruses are classified according to differences in their surface proteins. Currently, a total of 18 different antigenic variants of the hemagglutinin (HA) and 9 different neuraminidases (NA) have been identified (11–13). This classification is based on the antigenic differences elicited by the different subtypes, since specific HA and NA antibodies will typically

not cross react with other subtypes (1). The HA is key in the determination of the host range of influenza viruses. The HA recognizes the sialic acid (SA) receptors on epithelial cells and has different receptor binding specificities (14). While mammalian adapted influenza strains mainly recognize SA receptors bound to galactose by α2-6 linkages, avian adapted strains recognize SA bound by α2-3 SA linkages (1). In humans, the major site of replication of Influenza A is the respiratory tract, rich in α2-6 SA receptors. Conversely, in birds the virus replicates predominantly in the digestive tract that contains only by α2-3 SA. However, even though the lower respiratory tract of humans also contains α2-3 SA, it is harder for avian like influenza virus to reach, hence, viral pneumonias caused by avian like viruses in humans are rare (14,15).

The main forces behind Influenza evolution and diversity are: 1) frequent mutations introduced into the viral genome by the viral RNA dependent polymerase, 2) genetic constrains forced upon viral antigens by host immunity, 3) the capacity of the virus to undergo successful gene reassortment and 4) gene flow (4,11,16). Genes that code for surface antigens are under the greatest evolutionary pressure from the immune system of their hosts and exhibit constant genetic drift. Surface proteins are therefore prone to higher evolutionary rates and are also more susceptible to reassortment, since progeny viruses equipped with new surface proteins are more likely to exhibit immune evasion. However, internal genes are less likely to undergo antibody mediated selection, and have therefore more time to develop host specific adaptations. Due to the segmented genome of influenza, much of the evolutionary changes in this virus can be explained by genetic reassorment, when two or more viruses exchange gene segments during coinfection. In terms of reassortment, protein to protein interactions may have an important impact on the progeny's fitness, leading to new viral variants that may not have an increased fitness unless linked to a functionally compatible segment (1). For a virus to infect, replicate and transmit efficiently

in a different host, it must acquire a set of host specific adaptations. Hence, host specific evolution of viral proteins may select for a specific kind of virus, since the loss of host adapted genes can reduce the overall fitness of the progeny virion. These adaptations range from increased HA binding to host surface proteins, interaction of viral ribonucleic and polymerase protein complexes with host specific nuclear import factors, polymerase adaptation to host specific body temperature and viral evasion of host specific immune responses (4,17). Finally, the availability of gene segments due to gene pool isolation also contributes to the evolution of influenza (1,18).

Avian Influenza

Avian influenza (AIV, Influenza A) is carried mostly by wild birds in nature and are therefore known to be the natural reservoirs of influenza A (19). The transmission of AIV in wild birds occurs trough the fecal oral route and the main replication site in ducks is the intestinal tract, with an important amount of virus shed through feces (1). There are currently 16 HA and 9 NA subtypes circulating in avian populations (20). AIV can also be further subdivided according to differences in their pathogenic phenotypes in poultry. Infection with highly pathogenic influenza (HPAI) causes mortality approaching 100% in chickens. In contrast, infection with low pathogenic avian Influenza (LPAI) causes little or no disease, unless exacerbated by secondary infection or poor environmental conditions (21). LPAI viruses have been present in natural environment for several hundreds, if not thousands of years. They cause mostly asymptomatic infection and therefore their impact on human health is minimal and no need exists to control them in wild birds. The importance in control however does occur when these viruses are introduced in man-made settings or human populations, where host and environmental conditions can lead to the raise and establishment of HPAI strains (22,23).

Even though the virulence of AIV isolates is a polygenic trait, its major virulence factor is related to the presence of a polybasic cleavage site found in the HA precursor, HA0. Due to this difference, LPAI viruses are host-enzyme restricted and can only be cleaved in anatomic structures rich in trypsin-like enzymes, i.e., the intestinal and respiratory tract. In contrast, HPAI viruses can be cleaved by ubiquitous host proteases that recognize the polybasic cleavage site. This characteristic allows HPAI viruses to replicate systemically, resulting in severe disease and death. It has been shown that a polybasic cleavage site can arise as a result of gene insertion, gene substitution or due to recombination with other gene segments (19,24). Even though human infection with AIV are rare, the high fatality rate of up to 60% associated with some subtypes has prompted a worldwide display of AIV surveillance in hospitals, poultry industry and in wildlife. Nevertheless, the factors that determine if an avian influenza virus gains the ability to efficiently transmit between humans and become a pandemic, are still poorly understood (6,25).

So far, it is known that AIV prevalence in wild birds varies with geographical location, bird species and time of the year. *Anseriformes* and *Charadriiformes* are known to be the main carriers of AIV in the wild (1). Recently, it has been established that waders from the *Charadriidae* and *Scolopaciae* families carry a wider range of AIV subtypes than gulls and terns, but less diversity than ducks. Other bird species like geese, swans, cormorants and passerines have also been occasionally described as carriers of AIV. Nevertheless, their importance as reservoirs has not been established (20). Also, recently hatched birds have a much greater probability of testing positive for AIV. Likewise, female birds are thought to be less likely to carry AIV, according to one study (26).

Surface water of AIV contaminated lakes and ponds have also been considered a source of infection for birds, hence acting as long and short-term source of AIV infection for both domestic

and wild animals (27). This viral carrying capacity is mediated trough water temperature and chemistry. Viruses are shown to be the most stable at slightly basic pH (7.4-8.2), low temperatures (less than 17 ˚C) and fresh up to brackish salinity (up to 20 ppt) waters, conditions usually found in lakes, ponds, wetlands and estuaries. At ideal conditions, studies suggest that AIV would even be able to subsist without a host in the Northern U.S. and Canada between migration seasons. This indicates that water can act as a reservoir for year-round transmission of AIV (28).

Avian Influenza Surveillance

Avian influenza virus has been known to circulate in wild birds since the discovery of AIV in Common Terns (*Sterna hirundo*) in the early ’60s. Ever since, efforts have been aimed at identifying potential reservoirs of the disease (19,29). However, it was not until the HPAI outbreak of H5N1 in Hong Kong in 1997 and its consequent spread through large parts of Southeast Asia and the Middle East during the first years of the 21st century, that AIV surveillance was given priority as a tool to monitor for potentially pathogenic AIV strains around the globe (30).

AIV is found ubiquitously among wild birds in North America and Eurasia. Studies reveal the presence of all AIV subtypes in these locations (20,30,31). However, widespread surveillance efforts elsewhere are missing. Oceania, Africa, South America and Antarctica are, in decreasing order, still the most surveillance neglected areas (32). Nonetheless, data suggest that subtype diversity is similar across the globe (20). Recent surveillance reports from Australia and South America suggest the existence of phylogenetically distinct AIV lineages compared to Asian and North American strains (33,34). Nevertheless, much information in this regard is still missing from Africa and South America (20,35). Exhaustive surveillance efforts in North America and Europa of wild aquatic birds have determined that AIV prevalence is the highest in early fall and summer.

This is associated with higher population densities due to pre-migratory congregation and due to a higher proportion of susceptible AIV naïve fledglings. The prevalence of AIV in this age group can be up to 30%, hence targeted surveillance at juvenile birds during this time period can significantly increase the detection success (11,20).

Many *Anseriformes* and *Charadriiformes* are known for their yearly migration patterns, usually following determined flyways between their breeding and wintering grounds. However, within these migration flyways, certain stopover locations are shared spatiotemporally by different bird species with diverse migration patterns, thus offering the possibility for birds to transmit AIV to new areas of the world (20,31). Intercontinental transmission of genes is primarily mediated through shorebirds, since little connectivity exists between duck species that migrate between hemispheres or between North America and Eurasia. Nevertheless, an important sampling bias towards ducks makes it still hard to assess (36). Still, the specific species contribution in migration to the dissemination of AIV around the globe is still poorly understood. The Blue-wing teal (*Anas discors*) has been attributed as an important trans-hemispheric disperser of AIV in the Americas. This North American duck species that does yearly migrations to its wintering grounds; from northern Canada through the southern United states and Central America up to the Orinoco basin in South America (37,38). Phylogenic studies performed on viral isolates obtained from Blue-wing teals in Guatemala have yielded only North American origin viruses, indicating that this species is a major contributor to the ecology of AIV in central America (39). Nevertheless, AIV has not been to date isolated from this particular species in South America, raising question whether other key players in AIV dispersion might be involved.

Traditionally, AIV surveillance was performed by the collection of wild bird or poultry cloacal and fecal samples and by direct virus isolation in embryonated eggs or Mardin-Darby canine

kidney cells (40). This methods are very labor intensive and run the risks of false positives due to laboratory contamination, particularly during blind egg passages. Another drawback of this methodologies are that they are only able to detect replication capable viruses and the introduction of specific mutations associated with the viral growth substrate. Modern methodology in surveillance changed to real time RT-PCR detection of AIV, mainly because it facilitates the screening of large volumes of samples and allows the subsequent targeted isolation of candidate samples (41). In the recent years, the use of deep sequencing technologies allowed to obtain complete genomes without previous isolation, hence accelerating full genome AIV characterization even further (42).

Avian Influenza in South America

The epidemiology of AIV in South America is poorly defined. AIV was first isolated in 2001 from wild birds during a surveillance program conducted around Lake Titicaca in Bolivia. Since then, AIV isolates have been obtained from Peru (2007), Brazil (2008), Argentina (2007 and 2008), Chile (2002, 2007-2008) and recently from Colombia (2012) (43–49). Nevertheless, too little information has been gathered to provide a clear understanding of the ecology and distribution of AIV in the American sub-continent. To date only 14 different HA and NA combinations have been described in South America. In contrast, the North American Atlantic Flyway, Europe and Asia, have 94, 91 and 84 unique combinations, respectively (50). Given the worldwide distribution of AIV and its transmission through wild birds, my studies will test the hypothesis that a high diversity of potentially pathogenic variants of AIV are currently circulating in many areas of South America and weather these viruses are related to each other.

The Chilean and Colombian territories form part of North American flyways, bird migration routes that connect most of North America with Central America, the Caribbean and South America (20,36). Colombia in particular sees yearly migration of birds of all over the United States and Canada (38). Chile forms predominantly part of what is known as the pacific migratory flyway, a migration route that stretches from the furthermost tip of Alaska to the South American Patagonia, although some influence of birds that regularly migrate though the Atlantic flyway can be seen in the southern Patagonia of Chile (20). While intense surveillance efforts in North American wild birds have made and the bird migration dynamics and viral reassortment rates of AIVs between these flyways are well understood, influenza surveillance along the southern hemisphere section of these flyways has been limited (37). In 2002, Chile became the first country in South America to have confirmed isolates of HPAI, during an outbreak at an industrial broiler chicken farm. This outbreak was preceded by the detection in June of the same year of LPAI in turkeys in a nearby farm and confirmed the potential of LPAI to become HPAI when passaged in poultry (23,47). Interestingly, phylogenetic analysis of a LPAI H7N3 AIV obtained a year earlier in Bolivia, showed that both viruses where closely related with similarities in the HA, NP, PA, PB1 and PB2 segments. This suggests a common relative between both isolates and supports the hypothesis that the virus in Chile was introduced by wild birds (23,51). Up to 2009, only a two other AIV have been described in Chile: an H5N2 and an H13N2 obtained from shorebirds (46). On the other hand, studies performed in Colombia have so far only documented the presence of LPAI H5N2 and antibodies against H9N2 AIV (48,52). Phylogenic analysis of the Colombian H5N2 viruses shows that they are closely related to North American viruses circulating in wild birds (48).

Overall, previous studies performed in South America have suggested the existence of a phylogenetically unique clade of AIV in the sub-continent, but the geographical extent of the North- and South American lineages is unknown, as is the rate of exchange of gene segments between them (35,45,53). As evidenced by recent studies performed in Colombia, Brazil and Peru, the predominance of the North American lineage seems to be of major importance in South America up to the Tropic of Capricorn (48,49,54), north of were South American lineage gene segments are hard to find. Nevertheless, the sympatric existence of genetically distinct lineage of gene segments can provide a larger genetic pool for potential reassortment events and the subsequent generation of novel subtypes (35).

There are unique characteristics in Chile in terms of poultry and swine production practices compared to the rest of South America. In Chile, these market sectors are very industrialized, operating both breeding and processing units with high biosecurity standards. Moreover, live animal markets are uncommon. However, in rural areas it is still possible to find small size backyard breeding farms for household consumption of eggs and meat. These rudimentary production settings are often exposed to wild birds, the sanitary conditions are frequently poor and there is an increased risk of disease transmission to humans (21,55). However, no reports of AIV have been documented in backyard poultry in Chile to date. Therefore, an aim of this thesis was to prove that AIV is circulating in backyard poultry in Chile and to establish if circulating strains pose a threat to animal and human health. Colombia shares several characteristics in terms of poultry production with Chile, but differs in that the presence of live animal markets is very common throughout cities and villages. Previous reports have documented the circulation of AIV in backyard poultry in Colombia, but no subtype information was obtained (48). Hence, another goal of this research was to obtain isolates from poultry in Colombia and characterize them in

order to draw comparisons to other viruses circulating elsewhere in the continent. Considering that AIV remains understudied in the region, these studies intend to fill a gap in knowledge and potentially have important public health implications.

Chapter introduction

Chapter 2 was submitted to *Research in Veterinary Science* at the end of 2015 and is currently under revisions. In it, I demonstrated the circulation of influenza A in backyard poultry and swine in the central region of Chile. This is the first time AIV has been documented to circulate in small farms in Chile. I also described the genetic characteristics of the first H12 sequence obtained from domestic animals in South America to date.

Chapter 3 will be submitted to *Emerging Microbes and Infections* in May 2016. In this chapter I described the first detection and isolation of AIV in a live animal market in South America. In March 2015, active surveillance efforts at a live animal market in Medellin, Colombia, yielded two H11N2 AIVs and a peak influenza A prevalence of 17% in poultry. In the chapter 3 I described the epidemiological circumstances surrounding these findings, as well as the genetic and phenotypic characteristics of the isolates.

Chapter 4 will be submitted to *Journal of Virology* in summer 2016. In this chapter I demonstrated the ample circulation of AIV in waterfowl and shorebirds in Chile trough AIV surveillance, carried out between 2012 and 2015. This study accounts for the richest collection of AIV in Chile to date. Through it, I described the isolation of potentially pathogenic subtypes and the epidemiological, genetic and phenotypic characteristics of these isolates.

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Chapter 2

INFLUENZA VIRUSES IN BACKYARD POULTRY AND SWINE IN CENTRAL CHILE

Pedro Jiménez-Bluhm, Francisca Di Pillo, Justin Bahl, Jorge E. Osorio, Stacey Schultz-Cherry and Christopher Hamilton-West

Abstract

Backyard productive systems (BPS) are recognized as the most common form of animal production in the world. However, frequently BPS exhibits inherent biosecurity deficiencies, and could play a major role in the epidemiology of animal diseases and zoonoses.

The aim of this study was to identify if Influenza A viruses (IAV) were infecting backyard poultry and swine in central Chile. Serologic testing to detect antibodies was performed on 636 samples from poultry and swine in the region of LGB O´Higgins. The seropositivity rate in poultry level was 3.5% (CI: 2%-5%), and at BPS level was 12.7% (CI: 6.2%–19.9%). For pigs the seropositivity was of 1.6% (CI: 0%-3.2%), while at BPS level was 2.2% (CI: 0%-5.4%).

In Valparaiso and Metropolitan regions, a real-time reverse transcriptase PCR assay was used to detect influenza virus matrix gene. The positivity rate at poultry level was 3.84% (CI: 3.2%–4.5%), and at BPS level ranged from 16% (CI: 1.2%-28.9%) during winter 2012 to 95.2% (CI: 82.8%-99.6%) during fall 2014. Furthermore, an H12 hemagglutinin was obtained from a domestic Muscovy duck (*Cairina moschata*) by PCR amplification.

This study represents the first report of IAV circulating in BPS in Chile, and provides evidence of seasonality of the virus, with higher level during summer and fall. These results highlights the need for improve surveillance of IAV in backyard populations, to get more understanding of its epidemiology, considering the interaction of domestic animals, wild birds and the people in theses farms.

1. Introduction

Influenza A viruses (IAV) are considered a threat for both, public and animal health worldwide (1). The infection by IAV causes annual regional epidemics worldwide and sporadic pandemics with high mortality rates. Influenza pandemics usually occur after a periodic exchange of viral genes among human, swine, and wild and domestic bird adapted strains (2, 3). Wild birds are frequently infected with avian influenza viruses, and wild aquatic birds are considered the primary virus reservoir (4, 5). To date, more than 80 different combinations (HA and NA) of influenza viruses have been isolated from wild birds (6-8) and, as generally asymptomatic virus carriers, they have the potential to spread IAV between countries or even continents during their migration (7, 9). Complementarily, poultry production practices, trade of poultry, poultry products, and wild birds, considering legal and illegal activities, have been recognized as pathways by which avian influenza virus can spread locally and worldwide (10-13).

Smallholder production is practiced by most rural households throughout the world (FAO, 2010). This term refers to the many diverse forms of production found in communities of small-holders, being one of them the backyard productive systems (BPS). Although the term BPS is widely used, there are still difficulties when defining if a production system can be considered as such since there is no single definition due to the many factors that can make a productive system considered as a backyard, such as flock size where usually few animals are bred (less than 100 poultry and 10 pigs), biosecurity levels, which are generally absent and with practices such as keeping, handling or eating sick animals; and productive purposes, where the primary objective is to provide food for the families and sometimes complementarily generate revenues from animals/products sales (14, 15).

BPS represent more than 150 thousand farmers, breeding more than 3.7 million of poultry and more than 400 thousand pigs in Chile (14). The majority of BPS do not apply basic hygiene and biosecurity measures, and may not have knowledge about many animal diseases, therefore, sick animals may be handled, sold, slaughtered and consumed without considering that the infections that made an animal sick may also potentially be harmful to man (16, 17). Additionally, BPS represent the interface where interaction between domestic animals, wildlife and humans may occur (14). These conditions may increase the susceptibility of BPS to harbor and spread infectious pathogens such as influenza A virus (18).

Little is known about IAV in BPS worldwide. There is a lack of knowledge about prevalence and seroprevalence, subtypes circulating and their risk for public and animal health, hence the aim of this study was to identify if IAV were infecting backyard poultry and swine in central Chile, and to characterize circulating strains.

2. Materials and methods

2.1 Study area

This study was carried out in central Chile, including Valparaiso, Metropolitan and Libertador General Bernardo O'Higgins (LGB O'Higgins) regions. A convenience sampling method was carried out, based on the number of registered BPS in a local government subsidized development program, owning poultry and swine. BPS were defined as rural households units having up to 100 poultry (14) and up to 50 swine.

2.2 Sampling and samples analysis

During spring-summer 2012 and autumn-winter 2013, 636 blood samples were collected for serology in LGB O'Higgins Region. 3 - 5 mL of blood were collected from the brachial vein of each bird and from the marginal ear vein of each pig, and then placed into a 6 mL vacuum tube. Serum was obtained by centrifugation at 1,300g for ten minutes. The samples were kept at 4ºC during sampling and stored at -20ºC until analysis, at the Infectious Disease Laboratory, Faculty of Veterinary Science - University of Chile.

Identification for antibodies to AI virus in chickens' sera was carried out using the IDEXX AI Ab Test (Se: 95.4 and Sp 99.7), but for sera of all other species, the IDEXX Influenza A Ab Test was used (Se: 95.4 and Sp 99.7 for poultry, and Se: 95.3 and Sp: 99.6 for pigs). Plates were read using an INMUNSKAN Plus (BDSL) microplate reader.

3,197 cloacal and tracheal swabs were collected from poultry, to identify the presence of IAV, in Valparaiso and Metropolitan regions in four seasons (winter 2012, summer 2013, spring 2013 and fall 2014). Samples were collected using disposable sterile swabs and stored in cryovials containing 1 ml Universal Transport Media, UTM^{TM} (Copan Italia S.P.A). RNA extraction and real-Time PCR analysis of the samples was performed at facilities located at St. Jude Children's Hospital, in Memphis, TN, USA. Samples were kept at 4ºC during sampling and stored at -70ºC until analysis. Viral RNA extraction was performed using 50 µl of sample loaded on a Kingfisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA). The Ambion MagMax-96 AI/ND viral isolation kit was used for RNA extraction (Life Technologies Corporation, Grand Island, NY, USA). Sample screening was done by RRT-PCR (Bio-Rad CFX96 Real-Time PCR detection System). Specific primer/probes for the influenza matrix gene, taken from WHO/CDC protocol were used for the RRT-PCR reaction (WHO, 2009). Positive samples

under a RRT-PCR threshold value of 37 were passaged through nine day old embryonated chicken eggs as described by Lira, Moresco (19).

Single stranded DNA was obtained using SuperScript Vilo™ (Life Technologies Corporation, Grand Island, NY, USA). Amplicons were obtained using Q5 High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA, USA) using universal oligonucleotide primers, as described by Hoffmann, Stech (20). Further sequencing was performed by Sanger sequencing at the University of Wisconsin-Madison Biotechnology Center.

2.3 Phylogenic analysis

All publicly available avian origin H12 sequences at the Influenza Virus Resource at NCBI greater than 1500 base pairs originated in North America, South America, Europe and Asia (n=174) were used for phylogenic analysis. BEAST version 1.8.2 was used for the analysis (21-23). A HKY85 substitution model was applied and we used time-stamped sequence data with a lognormal relaxed-clock Bayesian Markov chain Monte Carlo (MCMC) method. For each analysis, the Bayesian skyline coalescent tree prior model was used (10 groups). The starting tree was selected randomly. We performed four independent analysis of 50 million generation each. We then combined the rounds after removing a 10% of burn-ins for each separate analysis and sampled every 15000 generations for a total of 12000 trees and parameters. FigTree version 1.4.2 was used for visualization of the annotated phylogenetic tree. The timing of the introduction of the H12 subtype into Chile was estimated by analyzing the times-scaled maximum clade credibility (MCC) tree.

2.4 Data analysis

A database was created in Excel (MS Office 2010). Descriptive analysis and graphical representations were performed in using Infostat® statistical software. A logistic regression model (24) was performed to identify assosiation in risk of influenza virus and different sampling seasons.

2.5 Biosecurity and ethics statement

All sampling activities and protocols were approved by the ethics and biosecurity committee of Faculty of Veterinary Science (FAVET), University of Chile, and by the Chilean National Commission for Technological Research (CONICYT).

3. Results

3.1 Seroprevalence influenza A virus

A total of 509 poultry sera samples were obtained from animals of different ages and species, including domestic chickens (*Gallus domesticus*), ducks (*Anas platyrhynochos*, *Cairina moschata*), turkeys (*Meleagris gallopavo*) and geese (*Anser anser domesticus*), from 113 BPS (Table 1). Chickens were the only species of poultry where seropositive birds were identified, with 18 positive samples form 14 BPS. Thus, the positivity rate of AI in domestic birds was 3.5% (CI: 2% - 5%), while the positivity rate at BPS level was 12.7% (CI: 6.2% – 19.9%). Seropositive birds by ELISA belonged to BPS located in Navidad, Litueche, Las Cabras, Paredones and Pichilemu counties (Figure 1). In each positive BPS, only one sampled bird was seropositive, except in Las Cabras, where two chickens were seropositive in the same BPS and 3 chickens were seropositive

in another BPS, as well as in Paredones, where the same BPS presented 2 seropositive chickens (Table 1). Serum samples that were positive by ELISA were referred to Official Chilean Veterinary Service (SAG) for confirmation by AGID. All samples where negatives to these test, discarding the presence of an AI outbreak.

As for pigs, a total of 127 samples were obtained (89 BPS), of which 2 were seropositive. The seropositivity rate of IAV in pigs kept in BPS was 1.6% (CI: 0% - 3.2%), while the positivity rate at BPS level was 2.2% (CI: 0% - 5.4%). Seropositive pigs were detected in BPS located in the counties of Placilla and Navidad (Figure 1 and Table 1).

3.2 Influenza A virus identification

For RRT-PCR analysis, 63 BPS were sampled during different seasons, and 3,197 samples were taken. The positivity rate at poultry level was 3.84% (CI: 3.2% – 4.5%), ranging from 0.7% (CI: 0.1% -1.4%) during winter 2012, to 13.5% (CI: 10.9% - 15.6%) in fall 2014. On the other hand, at BPS level the positivity rate ranged from 16% (CI: 1.2% - 28.9%) during winter 2012 to 95.2% (CI: 82.8% - 99.6%) in Fall 2014 (Figure 2 and Table 2). The logistic regression analysis showed statistical significance for positivity to influenza virus, associated to the sampling season. Samples collected during summer 2013 had an OR of 3.18 (CI: 1.19 - 8.51, $p= 0.02$), and those collected during fall 2014 had an OR of 21.9 (CI: 8.84 – 54.35, $p < 0.01$), indicating higher risk for influenza in BPS during summer and fall, when compared with samples collected during winter.

3.3 Virus sequence

One H12 hemagglutinin (HA) was obtained by PCR amplification from a domestic Muscovy duck (*Cairina moschata*) cloacal swab. This is so far the only H12 subtype obtained in the southern cone of South America. As typical for low pathogenicity IAVs, it had a PQIQNR/GLF cleavage site containing a single arginine (25). According to BLAST (http://blast.ncbi.nlm.nih.gov/) the closes hit for this HA is an H12N5 virus obtained in Alberta in 2003 (A/pintail/Alberta/49/2003) with a 94% sequence identity. Full coding sequence of the hemagglutinin was obtained and phylogenetically analyzed by Bayesian analysis. It becomes apparent that there are currently three lineages of H12 circulating in wild birds in North America to one of which this sequence belongs to: clades I, II and III. The time of most recent common ancestor (tMRCA) of these three clades is ~1987 (95% Bayesian credible interval 1980 – 1989). The H12 described in this study further diverged from clade III as recently as ~1999 (95% Bayesian credible interval 1997 – 2001) (Figure 3).

4. Discussion

There is a lack of timely surveillance of animal flu viruses, although it is recognized as an essential element for identifying pandemic threats, outbreaks detection, monitoring virus evolution, and to understanding risk factors that enable them to spread (26). This lack of surveillance becomes more evident in backyard animal populations (27). BPS are the most common form of animal production worldwide and its sustainability is considered a tool for poverty mitigation. Nevertheless, some pathogens could represent constraints for animal productivity, and a zoonotic risk for the people handling live animals and consuming their products. These risks were previously identified in BPS

in central Chile, based on production and value chain conditions (14). However, there was no information available on the presence of IAV infection in backyard poultry and swine populations in Chile.

This study presents the first report of influenza virus circulating in backyard swine and poultry in central Chile. Official surveillance activities had identified five avian influenza virus strains from 2002 to 2013 in Chile. Those strains were detected in wild birds or in large-scale poultry systems, and are as follows; HPAI H7N3 (2002, domestic chickens, Valparaiso region), LPAI H13N2 (2007, wild seagull, Atacama region), LPAI H5N9 (2008, wild seagull, Valparaiso region), pH1N1 (2009, domestic turkeys, Valparaiso region) and LPAI H4N8 (2011, domestic turkeys, Valparaiso region) (28-31). Moreover, swine influenza virus (IAV-S) is also recognized as a highly contagious infection of pigs. In Chile, is one of the most prevalent diseases in large swine production companies, with serological evidence of pH1N1, H1N1 and H3N2 infection (32). Recently (33) isolated subtypes H1N2, H1N1 and H3N2.

In South America little is known regarding influenza virus circulating in backyard poultry and swine populations. An 11% of seroprevalence of IAV was reported in poultry kept in BPS in Ecuador (34). However, a study in Argentina reported an absence of antibodies or virus circulating in BPS between 1998 and 2005 (35). For swine influenza, pH1N1 virus was isolated in Perú in backyard farms, including a seroprevalence ranging from 0% to 24% (36). Our results are similar in terms of seropositivity to findings reported in Ecuador, but when considering positive results for RRT-PCR, positivity levels are higher and increase in summer and fall. These results provide evidence of seasonality in the presentation of influenza in BPS, considering fall season in which more positives were presented. This could be due to increased virus load in the environment after that migratory bird have nested in the country and then return to their origin sites. These results

are consistent with a study conducted in the USA where increased presence of influenza in commercial turkeys in summer and fall was described (37). Moreover, studies in China have described seasonality in the presentation of avian influenza in fall-winter (38) while other described it in winter and summer (39). Nevertheless, more studies are needed to understand circulation dynamics of influenza virus in backyard animals.

In terms of the obtained subtype, phylogeny indicates that there has been at least 15 years of unsampled diversity of this particular subtype in Chile. Clade III also contains H12 sequences from shorebirds obtained in Peru in 2008, being these three sequences the only ones so far described for this subtype in South America. The particularity of the finding of this study lies in that H12 was obtained from a domestic duck, being this to the author's knowledge the first time that this subtype has ever been obtained from a domestic animal in South America. This may also indicate that AIVs circulating in backyard poultry in Chile may represent a smaller pool of subtypes owed to the population isolation that is subjected upon domestic animals due to limited animal movement and trade. No reports of outbreaks of highly pathogenic H12 AIV have been reported so far, which indicates that the pathogenic potential of this particular subtype may be limited. Nevertheless, the effect of the infection in domestic animals by this particular subtype is unknown.

The probability of introduction and spread of a disease in a country is determined by a network of factors, which, when referring to BPS, gain greater relevance (14, 40). Poultry and swine production are characterized by the diversity of production systems, with different scales of production, biosecurity measures and entry and exit of products. Thus, intensive production systems and BPS coexist, having different characteristics between them. However, both keep animals that are susceptible to the same diseases, including those caused by influenza virus (40,

41), suggesting the need of coordinated efforts to avoid outbreaks affecting the public health and the economy of the country.

5 Conclusions

Influenza virus circulates in backyard farms in Chile, representing a risk for both public and animal health. The results of this study highlight the need for improve surveillance of influenza virus in backyard populations, and also to get more understanding of the epidemiology and factors that could explain seasonality of influenza in backyard animals, in order to define preventive measures to reduce the risk for the animal and public health, and the economy of the country.

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Author Contribution:

Jimenez-Bluhm, P (self) Designed sampling efforts and performed screening for the detection of Influenza A. Performed phylogeny. Organized and conctructed figures. Co-wrote manuscript with CHW.

Di Pillo, F Collected and screened samples for serology.

Bahl, J Assisted in phylogenic analysis.

Osorio, JE Assisted in designing sampling strategies. Assisted in preparation of manuscript.

Schultz-Cherry, S Assisted in designing sampling strategies. Assisted in preparation of manuscript.

Hamilton-West, C Designed serosurveillance. Organized and conctructed figures. Co-wrote manuscript with PJB.

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Table 1

Results of sampling for Influenza A antibodies detection in backyard poultry and swine, by county of the LBG O'Higgins region.

County	Sampled BPS (N)	Number of sampled animals					IAV seropositive birds			IAV seropositive pigs		
		Chicken	Duck	Turkey	Goose	Pig	N	%	CI	N	%	CI
Placilla	20	99	6	3	0	35	0	-	-	1	2.86	0 - 9.2
Nancagua	3	12	2	0	0	5	0	-	-	0	-	-
Malloa	4	15	0	0	3	8	0	-	-	0	-	-
Navidad	24	96	6	8	2	30	1	0.89	0 - 2.7	1	3.33	0 - 9.6
Litueche	5	12	0	1	0	10	1	7.69	0 - 21.7	0	-	-
Las Cabras	15	53	7	1	1	9	6	9.68	2.5 - 17.0	0	-	-
Paredones	12	35	1	7	0	8	5	11.63	2.7 - 23.7	0	-	-
Chimbarongo	6	27	4	0	0	6	0	-	-	0	-	-
Pichilemu	19	83	4	0	0	11	5	5.75	1.2 - 10.9	0	-	-
Marchihue	5	8	8	5	0	5	0	-	-	0	-	-
TOTAL	**113**	**440**	**38**	**25**	**6**	**127**	**18**	**3.5**	**2.0 - 5.1**	**2.00**	**1.57**	**0 - 3.6**

Table 2

Results of sampling for Influenza A detection by RRT-PCR in backyard poultry and swine, by season and county of the Metropolitan and Valparaíso regions.

Season	***County***	***Number of sampled BPS***	**IAV positive BPS by RRT-PCR**			***Number of samples taken***	**IAV positive samples by RRT-PCR**		
			N	**(%)**	**CI (%)**		**N**	**%**	**CI (%)**
Winter 2012	Talagante	16	2	12.5	0 - 28.3	305	2	0.7	0 - 1.6
	El Monte	9	2	22.2	0 - 47.8	244	2	0.8	0 - 1.9
	Peñaflor	6	1	16.7	0 - 45.5	156	1	0.6	0 - 1.8
	Total	**31**	**5**	**16**	**1.2 - 28.9**	**705**	**5**	**0.7**	**0.1 - 1.4**
Summer 2013	Talagante	14	4	28.6	3.7 - 57	283	7	2.5	0.7 - 4.3
	El Monte	8	2	25	0 - 49.4	299	3	1	0 - 2.2
	Peñaflor	10	5	50	11.6 - 76.4	278	9	3.2	1.1 - 5.1
	Melipilla	1	1	100	100 - 100	41	1	2.4	0 - 7.7
	Total	**33**	**12**	**36.8**	**21.3 - 52.2**	**901**	**20**	**2.2**	**1.2 - 3.1**
Spring 2013	Talagante	9	1	11.1	0 - 51	193	1	0.5	0 - 1.5
	Peñaflor	9	0	-	-	182	0	-	-
	Padre Hurtado	8	4	50	0 - 68.4	281	6	2.1	0.6- 3.9
	Melipilla	1	0	-	-	37	0	-	-
	San Antonio	6	2	33.3	0 - 68.4	248	2	0.8	0 - 1.8
	Total	**33**	**7**	**21.6**	**7.2 - 33.8**	**941**	**9**	**1**	**0.4 - 1.6**
Fall 2014	Talagante	10	9	90	63.1 - 99.3	204	29	14.2	9.9 - 18.1
	Padre Hurtado	8	8	100	100 - 100	227	29	12.8	8.5 - 17.1
	San Antonio	6	6	100	100 - 100	219	30	13.7	8.9 - 18.4
	Total	**24**	**23**	**95.2**	**82.9 - 99.6**	**650**	**88**	**13.5**	**10.9 - 15.6**

Figure 1.

Spatial distribution of sampling results of Influenza A seropositivity in backyard poultry and swine.

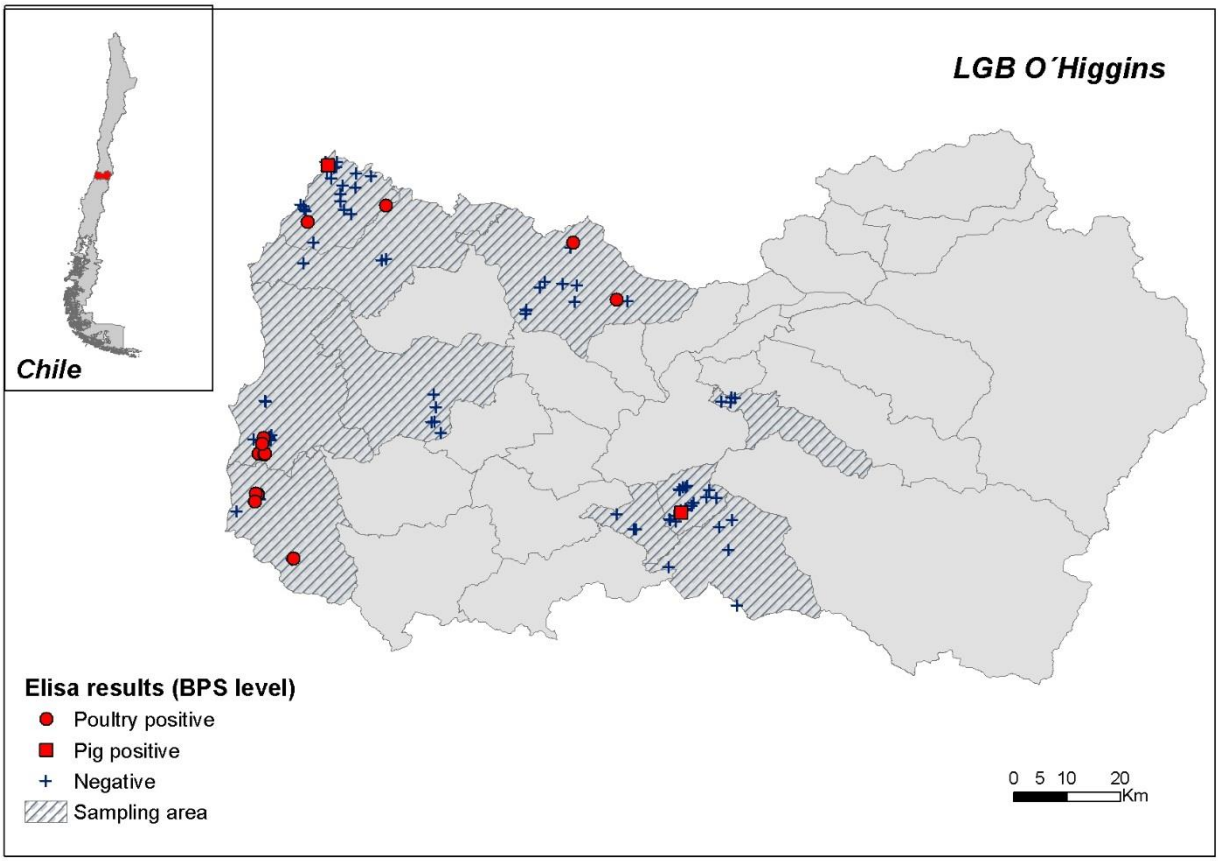

LGB O´Higgins
Chile
Elisa results (BPS level)
Poultry positive
Pig positive
Negative
Sampling area
0 5 10 20
Km

Figure 2.

Spatial distribution of positive results of Influenza A by RRT-PCR in backyard poultry and swine.

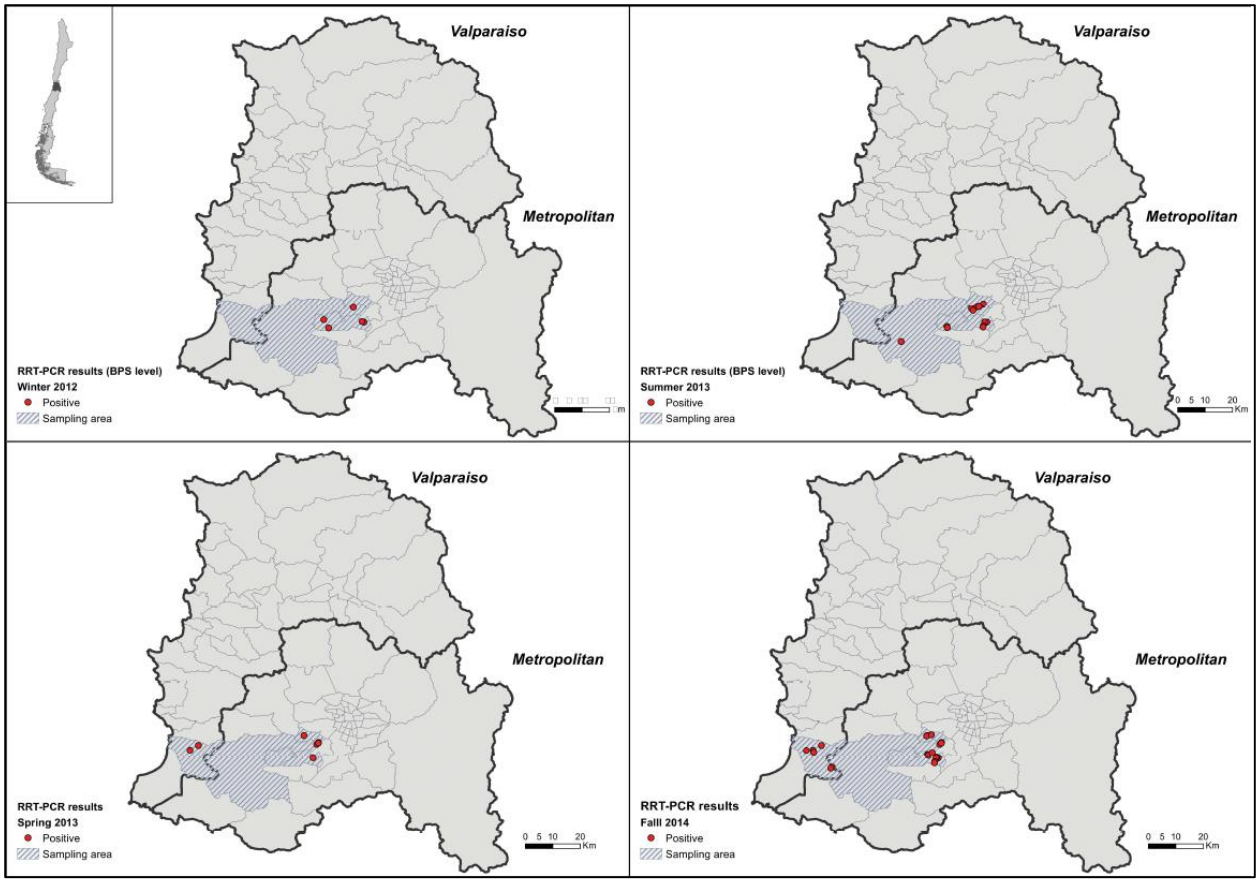
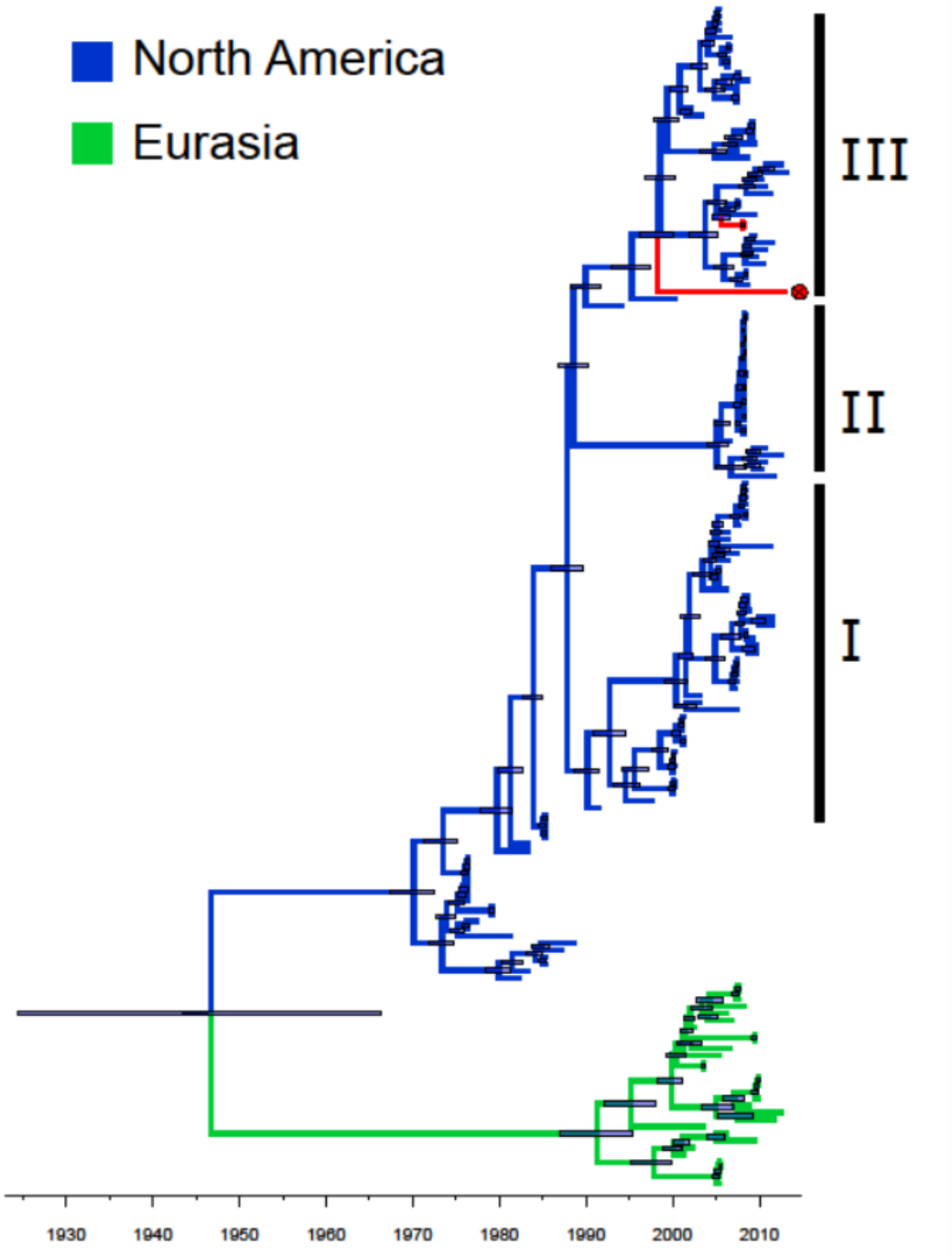

Valparaiso
Metropolitan
RRT-PCR results (BPS level)
Winter 2012
Positive
Sampling area
Valparaiso
Metropolitan
RRT-PCR results (BPS level)
Summer 2013
Positive
Sampling area
0 5 10 20
Km
Valparaiso
Metropolitan
RRT-PCR results
Spring 2013
Positive
Sampling area
0 5 10 20
Km
Valparaiso
Metropolitan
RRT-PCR results
Falll 2014
Positive
Sampling area
0 5 10 20
Km

Figure 3. Phylogenic relationship between North American and Eurasian H12 segments

Time scaled Bayesian MMC tree for the H12 segments (n= 174 sequences). In red are represented the three known introductions of this subtype into South America (Chile, Peru). The symbol indicates the H12 obtained in Chile. The currently circulating clades of H12 subtypes in wild birds in North America are denoted by roman characters. Node bars indicate the 95% Bayesian Credible interval.


North America
Eurasia
III
II
I
1930
1940
1950
1960
1970
1980
1990
2000
2010

Chapter 3

EMERGENCE AND SPREAD OF AN AVIAN H11 INFLUENZA VIRUS IN A COLOMBIAN LIVE ANIMAL MARKET

P. Jiménez-Bluhm, K.A. Ciuoderis, V. Cortez, E.A. Karlsson, C. Hamilton-West, J.E. Osorio and S. Schultz-Cherry

ABSTRACT

Live animal markets (LAMs) can be “hotbeds” for the emergence of new influenza viruses that occasionally spill over into humans. They are also culturally important for many Latin American countries, as they provide an essential source of food and trade for a significant part of the population. Yet despite the known role of LAMs in the spread and maintenance of avian influenza viruses (AIV), no studies to date have addressed the prevalence of AIV in LAMs in South America. To fill this gap in knowledge, active surveillance was carried out at a major LAM in Medellin, Colombia during 2015. During this period, we detected an H11N2 virus that asymptomatically spread through multiple bird species resulting in 17.0% of the birds testing positive at its peak. Genetically, the viral hemagglutinin (HA) and all other gene segments were of North American origin. Phenotypically, the H11 viruses displayed no known molecular markers associated with increased virulence in birds or mammals, had an α2,3-sialic acid binding preference, and caused little morbidity *in vivo*. However, the Colombian H11N2 virus replicated and transmitted effectively in chickens explaining the spread throughout the market. The genetic similarity to H11 viruses isolated from South American shorebirds suggest that the LAM occurrence, the first reported in South America, may have resulted from a wild-bird to domestic poultry spillover event and highlights the need for enhanced AIV surveillance in South America including in LAM workers, given that H11 viruses have been reported to cause human infections.

INTRODUCTION

Live animal markets (LAMs) around the world represent an important and traditional place for congregation and commerce, particularly in developing countries. Due to their role as a source of affordable, live or freshly slaughtered animals, LAMs are known to play an important role in the zoonotic transmission of pathogens, especially viruses (1–3). Spread of a virus within the market is often enhanced due to animals being kept in close contact and at high densities, increasing the risk of transmission between susceptible animals and people (4). Animals kept in LAMs can remain there for extended periods of time until sold and can consequently transform these markets into viral reservoirs, as infected animals can transmit virus to newly incorporated naïve ones, thus perpetuating and amplifying viral circulation (5). Additionally, LAMs are often part of a larger marketplace, potentially exposing people to zoonotic diseases that otherwise would not be in direct contact with infected animals (1).

Of particular importance, increased urbanization coupled with persistence of traditional trade practices have favored conditions for avian influenza viruses (AIV) to circulate in LAMs in Asia(2,6–8) . In the Americas, there have been reports of AIV in LAMs located in North America and the Caribbean, but no cases of AIV have been reported at LAMs in South America to date(9,10), likely due to minimal surveillance efforts (11). In addition, while a good amount of data is available on highly pathogenic AIV such as H5 (5,12,13), LAMs are also a source of low pathogenic AIV (LPAI) which can spread asymptomatically through poultry and are difficult to detect without routine surveillance (14–17). While LPAI subtypes H7 and H9 have regularly been detected in LAMs(8,18–20), other subtypes could be equally important, such as H11. H11 subtype AIVs are distributed worldwide (9,21–23), found primarily in wild ducks and shorebirds (24,25) and have been reported to cause human infections (26,27). However, they have been detected only

a handful of times in domestic birds at farms or LAMs, mainly in Asia and North America (6,9,17,28).

During active surveillance at the largest LAM in Medellin, Colombia, we isolated two H11N2 viruses from asymptomatic birds. Throughout this 8-month sample period in 2015, 17.0% of the screened domestic poultry in the market tested positive for AIV at the peak of a transmission the event. Characterization of the viruses demonstrated that they are most similar to viruses from North American migratory birds and to Chilean wild bird H11 viruses isolated in 2013. Given that this is the first report of AIV in a LAM in South America to date (9,10), coupled with the fact that there is very little information on H11 viruses in domestic poultry and that H11 viruses have the potential for zoonotic transmission (26,27), we assessed the risk of these viruses *in vitro* and *in vivo*. Phenotypically, the H11 viruses displayed no molecular markers associated with increased virulence in birds or mammals and had an α2,3-sialic acid binding preference. Colombian H11 viruses replicated and transmitted effectively in chickens, explaining the spread throughout the market, but caused little morbidity in Balb/c mice, indicating low risk for zoonotic potential. The genetic similarity to H11 viruses isolated from South American shorebirds suggest that the LAM occurrence may have resulted from a wild-bird to domestic poultry spillover. Given the widespread presence of H11 AIVs in wild birds throughout South America(23,29), these findings highlight the need for enhanced AIV surveillance in South America, especially in areas of high-risk, such as LAMs.

MATERIALS AND METHODS

Ethics statement

All animal experiments and field sampling activities were approved by the St Jude Children's Research Hospital Institutional Animal Care and Use Committee (IACUC). All sampling activities were carried out after verbal consent was obtained from the bird owners.

Sample site and collection

Sample collection (N=1160) was conducted between February and September 2015 (February, n=90; March, n=226; April, n=112; May, n=142; June, n=150; July, n= 72; August, n=209 and September, n=159) in a LAM in Medellin, Colombia. This is the only LAM that is consistently open to the public and a traditional place for people to obtain poultry and other groceries. There are 5 regularly established poultry sellers at the LAM, and around 2500 birds are available for sale at any given time. New birds are brought to the market weekly to bi-weekly, the majority of which are supplied by backyard poultry farmers. Poultry are sold mostly alive, but can be slaughtered, de-feathered and eviscerated at the LAM upon request. Samples from the LAM were collected from fresh environmental feces and cloaca of birds using single-use sterile swabs and placed in cryovials containing 1 ml Universal Transport Media, UTM^{TM} (Copan Italia S.P.A). Samples were kept at 4^{o}C for a maximum of 4 days then stored at -80^{o}C until analysis.

Screening and virus isolation

Following previously described methodology (30), viral RNA was extracted from 50 µl of sample using the Ambion MagMAX-96 AI/ND Viral RNA Isolation kit (Life Technologies Corporation,

Grand Island, NY, USA) using a Kingfisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, USA). Influenza M gene Real-time reverse transcription PCR (qRT-PCR) was performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA) and primers/probe as described (31). Samples with a cycle threshold value <38 were considered positive (32) and viral isolation in embryonated chicken eggs was attempted as described (33). Isolates were confirmed by hemagglutination assay (HA) and qRT-PCR and viral titers determined by Reed and Munch (34) with both 50% tissue culture infectious dose ($TCID_{50}$) in Madin-Darby canine kidney cells (MDCK) and by 50% egg infectious dose (EID_{50}). Viruses were stored at -80°C.

Virus Sequencing

Reverse transcription of viral RNA was performed using SuperScript Vilo™ (Life Technologies Corporation, Grand Island, NY, USA). Amplicons were obtained using Phusion High-Fidelity DNA polymerase (New Ingland BioLabs, Ipswich, MA, USA) with gene specific universal oligonucleotide primers as described (35). DNA was subsequently purified by agarose gel electrophoresis, using Zymoclean™ Gel DNA Recovery (Zymo Research Corporation Irvine, CA, USA). Full-length gene segments were ligated into the pCR™-Blunt II-TOPO® (Life Technologies Corporation, Grand Island, NY, USA) and amplified in HB101 *E. coli* strain (Zymo Research Corporation, Irvine, CA, USA). Smaller gene fragments produced using HA1134F/HA-NS 890R primers were sequenced directly after gel purification (36). Sequencing was performed by Sanger sequencing at the University of Wisconsin-Madison Biotechnology Center and at the St Jude Hartwell Center using segment specific primers (35,36). Host species were identified by PCR

barcoding using primers designed to amplify a ~700bp segment of the mitochondrial cytochrome-oxidase I (COI), obtained from AIV positive samples then sequenced as described (37). AIV gene segments and COI similarities were analyzed by BLAST (38).

Phylogenetic Analysis

Sequence assembly and manual edition was performed using BioEdit version 7.2.5 (39). Sequence alignments were generated using MUSCLE version 3.8.3 (40). Genome searches and obtainment of reference sequences for the alignments was done through the Influenza Virus Resource at NCBI (41). Phylogenetic analysis of the gene segments was performed using standard methods. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model using MEGA version 6.0 (42). The trees with the highest log likelihood are shown. In order to provide statistical robustness to each node, a bootstrap resampling process of 500 replicates was implemented. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

Assay strains

For in *in vitro* and *in vivo* assays, we used the following H11 clade viruses for comparison: A/duck/Memphis/546/1974 (H11N9, duck/Mem), A/ruddy turnstone/DE/544/2014 (H11N2, RT/DE), A/mallard/Alberta/315/2012 (H11N9, Mal/Alb), A/duck/England/1/1956 (H11N6,

Duck/Eng), A/mallard/Wisconsin/11OS4115/2011 (H11N9, Mal/WI), A/mallard/Mississippi/12OS361/2012 (H11N9, Mal/MS), and A/black necked stilt/Chile/2/2013 (H11N9, BNS/Chile).

In Vitro Replication

MDCK and A549 cells (ATCC, CCL-185) were cultured in Eagle's minimum essential medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 2 mM glutamine and 10% FBS (Gemini BioProducts, West Sacramento, CA) and grown at 37°C in 5% CO_2 in a humidified atmosphere. Viral replication studies were performed as described (30). Briefly, cells were infected at a multiplicity of infection (MOI) of 0.01 for 1 h at 37°C. Cells were washed three times to remove unbound virus and cultured in media containing 0.075% bovine serum albumin and 1 μg/ml L -1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Aliquots of culture supernatants were collected at 6, 24 48 and 72 h post-infection (pi) and immediately stored at –80°C. Viral titers were determined by $TCID_{50}$ in MDCK cells as described above.

Receptor Binding Specificity

Receptor affinity was determined using a solid-phase direct virus binding assay as previously described (43). Briefly, influenza viruses were bound to fetuin-coated plates at 4^{o}C overnight. Biotinylated glycans (α-2,3 or α-2,6 Sialic acids), Glycotech Corporation, Gaithersburg, MD) were added to influenza-coated plates at varying dilutions and incubated for a further 4 hours. Glycan binding was analyzed using HRP-conjugated streptavidin (Invitrogen, Carlsbad, CA) followed by TMB substrate (Sigma, St. Louis, MO) and plates were read at 450 nm on a Synergy 2 multi-mode

microplate reader (BioTek Instruments, Winooski, VT). Kd was determined by Linear Regression analysis using GraphPad Prism 5 software.

Animal Infections

6- to 8-week-old female BALB/c mice (n=11, Jackson Laboratory, Bar Harbor, ME) were lightly anesthetized with isoflurane and intranasally inoculated with 10^4 $TCID_{50}$ of virus in 25 µl PBS. Mice were monitored daily for sings of infection (body weight loss, hunched posture, ruffled coat, lethargy and dehydration) and weighed every 24 h (44). At day 3 and 6 pi, n= 3 mice were euthanized and nasal washes and lungs were harvested for viral titers by $TCID_{50}$. Chicken experiments were performed as described previously (30). Briefly, to determine virus shedding and pathogenicity after natural route infection, 8-week-old SPF chickens (n=5 per group) were inoculated with 10^6 EID_{50} of virus in 0.5 ml via intraocular, intranasal, and intratracheal routes and monitored daily for clinical signs of infection (labored breathing, body weight loss, diarrhea). One day post infection, naïve chickens (n=5) were introduced to simulate contact transmission in a market setting. To assess virus shedding, cloacal and tracheal swabs were collected every 48 h for 12 dpi. Swabs were stored in 1 ml (cloacal) or 0.5 ml (tracheal) viral transport medium at -70 °C for virus titration by determining 50% egg infectious dose (EID_{50}) in embryonated hen eggs.

Statistical Analysis

Logistic regression analysis was used to assess differences in AIV positivity across various bird species. A p-value <0.05 was considered statistically significant. All statistical analyses were performed with STATA statistical software, Version 13 (StataCorp, College Station, TX, USA).

RESULTS

Isolation of H11N2 viruses from Colombian LAM

Given the dearth of knowledge about AIV prevalence in South American LAMs, we initiated active surveillance in a popular, traditional LAM in Medellin, Colombia, in February 2015. Several bird species were tested (Table 1), with domestic ducks, chicken and turkeys being the most frequently sampled species. AIV was first detected in March when 3/226 birds tested positive (1.3%) by qrtPCR, peaked in April with 19/112 positive birds (17.0%), subsiding in subsequent months, indicating that the event was a self-contained occurrence. No positive samples were obtained after September 2015. Based on sampling data, guinea fowl and turkeys were more likely to be AIV-positive as compared to chickens (guinea fowl: OR=10.65, 95% CI: 2.82-40.31, $p<0.001$; turkey: OR=4.47, 95% CI: 1.10-18.15, $p=0.036$). No significant differences were observed with ducks, geese, or quail. No increase in morbidity nor mortality of poultry was reported to us by bird owners during the sample period, and we did not notice any clinical signs in poultry consistent with influenza infection, like ruffled feathers, diarrhea, decreased activity or respiratory distress. Two H11N2 viruses, A/Helmeted guineafowl/Colombia/1/2015 and A/Helmeted guineafowl/Colombia/2/2015, were isolated from different birds during the month of March. In addition, partial sequences of four additional H11N2 viruses where obtained from geese and guineafowls sampled during the month of April. Sequencing was attempted on all samples below cycle threshold value of 35(22), however no other AIV subtypes were detected during screening.

Phylogenetic and antigenic characterization of the H11N2 viruses

To begin to understand the genetic origin of the Colombian H11N2 viruses, we performed full-length sequencing on the viral isolates followed by phylogenetic analysis. In analyses of all gene segments, the two Colombian H11N2 viruses were more closely related to each other than to any other influenza virus, and they formed clusters significantly distinct from those of the other strains analyzed. All gene segments of both viruses clustered with AIVs belonging to a North American AIV lineage, rather than with viruses belonging to a South American or Eurasian lineage (Figure 1, Supplemental Figures 1-3). According to a BLAST search, the internal genes of the two H11N2 isolates had 98%-99% sequence similarity with those of their nearest relatives, all from North America (Table 2). Since both H11N2 viruses were virtually identical in all gene segments, only A/Helmeted guineafowl/Colombia/2/2015 (HGF/Colombia) was used in the subsequent assays.

We also obtained partial HA sequences from 4 additional positive LAM samples (Figure 2). The closest relatives were viruses we isolated in 2013 from wild birds in Chile (A/black necked stilt/Chile/1/2013 and A/black necked stilt/Chile/2/2013, 98% nucleotide similarity) and 2014 in Delaware Bay (A/ruddy turnstone/504/2014 H11N2 (99% nucleotide similarity), both viruses also belonging to the North American AIV lineage. Most of the other closely related HA sequences came from migratory birds in the Atlantic or Mississippi flyways (Figure 1). As expected, Colombian H11 viruses had a deduced amino acid sequence of PAIAT**R**/GLF at the multibasic cleavage site, indicating avian affinity and inability to replicate in the absence of trypsin. No molecular substitutions associated with mammalian host adaptation, like PB2 E627K or D701N(45,46) were found. HA receptor binding pocket residues (H3 numbering), at position 190, 225, 226 and 228 exhibited all avian-like amino acids that typically bind to α2,3-sialic acid receptors. Nevertheless, position 137 displayed a human-like adaptation by presenting an arginine

(47). Like the HA segment, the neuraminidase (NA) genes of the Colombian viruses were more similar to each other than to any other strain and clustered in the North American clade of N2 viruses containing long NA stalks (Figure 1). The nearest relative to both strains was identified by the N2 sequence of A/northern shoveler/California/3769/2012 H6N2 (99% nucleotide similarity). The NA and the PB1 gene segments also clustered with the highly pathogenic avian influenza (HPAI) H5N2 viruses circulating in the US in 2014-2015, supported by high bootstrap values (Figure 1, Supplemental Figure 3). Overall, sequence similarity was also high when compared to the H11N9 (BNS/Chile) obtained in South America, ranging between 87% and 99% (Supplementary Table S1). No changes associated with antiviral resistance were found (48,49)

Colombian H11N2 isolate binds to avian receptors and replicates in chickens

Given that H11 infection in poultry is uncommon (6,9,28) and that different domestic bird species were affected by this virus at the LAM , we performed a solid-phase glycan-binding assay and a transmission study in chicken in order to evaluate the risk this strain poses to poultry. Unsurprisingly, all of the tested H11 viruses had α-2,3 binding specificities with minimal binding to α-2,6 receptors, as expected of avian adapted viruses (Figure 3). Based on the α-2,3 binding specificities of the Colombian H11N2 virus and that it was originally obtained from asymptomatic poultry, we evaluated the pathogenicity and transmissibility of the virus in chickens. Groups of 8-week-old SPF chickens (n=5/virus) were inoculated by natural route with Duck/Mem, RT/DE, BNS/Chile and HGF/Colombia and monitored for clinical signs of infection. After 24 hours, naïve birds (n=5) were housed with infected animals to monitor transmission. Cloacal and oropharyngeal swabs were collected for 12 days post-infection (pi). Although none of the chickens exhibited clinical signs of disease, 100% of those inoculated with HGF/Colombia shed virus from the cloaca

by day 2 pi, with a peak virus titers ranging from $10^{4.5}$ to $10^{6.5}$ EID_{50}/ml at day 4 pi (Table 3). Chickens inoculated with the other H11 viruses also shed virus at similar titers at the peak of infection but cleared virus by day 10 pi while HGF/Colombia didn't clear until day 12. Similarly, oropharyngeal AIV titers of chicken infected with HGF/Colombia were detected, but peaked at only 10^3 to $10^{4.5}$ EID_{50}/m by day 2 pi. Further, the Colombian virus was the only one that transmitted to 60% of the contact animals by day 4 pi as measured by cloacal titers (Table 3). This transmission also explains the viral spread throughout the market.

Replication of the Colombian H11N2 viruses in vitro and in vivo

Given previous reports of human infection by H11 viruses (26,27), we performed *in vitro* and *in vivo* risk assessment in mammalian cell lines and mice. The H11 viruses had decreased replication in both MDCK (Figure 4A) and A549 (Figure 4B) cell lines as compared to mammalian control virus, A/California/04/2009 (CA/09; pdmH1N1). H11 viruses reached $10^{4.5}$ $TCID_{50}$/ml by 48 hour post-infection (hpi) unlike CA/09 that typically reached ~$10^{7.5}$ $TCID_{50}$/ml in MDCK cells (Figure 4A). Similarly, H11 viruses achieved only $10^{3.5}$ $TCID_{50}$/ml by 48 hpi as compared to $10^{5.5}$ $TCID_{50}$/ml by CA/09 in A549 cells (Figure 4B).

In mice, the majority of H11 viruses caused little to no weight loss and minimal viral titers were detected in the lungs at 3 dpi (Figure 5A, 5B). Interestingly, the Chilean H11N9 isolate (BNS/Chile) produced a sharp decline in weight loss reaching 30% by 7 dpi (Figure 5A). Since the H11N2 viruses did not bind to α2,6 receptors, had inefficient replication in mammalian cells and did not show signs of morbidity or produce mortality in mice, it was determined unnecessary to assess pathogenicity and transmissibility in a ferret model. In summary, the Colombian H11N2

viruses replicated poorly in mammalian cells and in inoculated mice, producing no signs of morbidity in the latter.

DISCUSSION

While the H11 subtype has been found globally in wild birds (21,24,50), few studies have identified H11 viruses from domestic poultry (6,9,28). During active surveillance in a LAM in Colombia, we observed an outbreak of LAIV and were able to isolate H11 viruses from two separate birds. The Colombian H11 viruses have many features expected of a wild-bird adapted AIV. Phylogenic and sequence analysis of all gene segments showed their similarity to wild bird viruses of North American origin, much like all other H11 viruses obtained in South America to date (23,29). The PB1 and NA genes clustered together very closely to gene segments of the HPAI H5N2 viruses circulating in the US and Canada between 2014 and 2015, indicating that partial reassortants of these HPAI viruses have been dispersed by wild birds throughout the American continent. However, unlike other poultry adapted AIVs, the N2 protein displayed a full-length stalk region(51,52). The conservation of the stalk sequence implies that there is still no significant adaptation of the H11N2 in *Galliformes* in spite of its widespread transmission at the LAM. This suggests that they were recently introduced by wild birds into poultry, very similar to HPAI H5N2 viruses isolated from North American poultry flock that did not display this adaptation (53). Interestingly, despite having the majority of characteristics of a wild bird virus, HGF/Colombia, efficiently replicated in poultry and was able to transmit to naïve contact animals, indicating a potential risk for poultry production in the region.

Epidemiologically, it is extremely difficult to determine the origin of the H11 viruses obtained at the LAM for several reasons. First, birds at LAM are received from commercial and

backyard poultry farms located throughout Medellin and the surrounding cities, but can occasionally be imported from other provinces throughout the country. Furthermore, birds are housed in close contact and sick animals are not separated from healthy ones. The combination of multiple sources of birds, constant influx of animals and close contact make it very hard to trace the origin of the infection. One possible explanation is that the virus was introduced at the LAM *in situ*. Doves and passerines feeding upon leftover grains are commonplace throughout the market and could act as potential carriers of AIV (54). Alternatively, many of the birds for sale at the LAM are raised in backyard flocks that are often exposed to wild birds (55), and could have therefore carried the virus to the LAM. Since this is the first study to observe AIV in a South American LAM, future studies need to be aimed at clarifying transmission dynamics. Importantly, they should involve sampling feral birds at the LAM as well as the screening of poultry on their arrival to the market. Environmental screening of cages, floors and equipment as well as abiotic factors, like temperature and humidity, could also provide important information as to whether these object contribute to the spread and maintenance of AIV in South American LAM. Our results also indicate that guineafowls and turkeys had higher odds to be infected by AIV at the LAM compared to Chicken and could therefore act as sentinel species in further studies.

Serological evidence of H11 infection in humans comes from several different sources, including North American duck hunters and wildlife professionals (27) as well as Lebanese poultry growers (26). These findings indicate that H11 viruses may have zoonotic potential; however, our studies performed on the Colombian H11N2 viruses proved inconclusive regarding risk of mammalian infection. H11 viruses grew inefficiently in mammalian cell lines and replicated poorly in the murine respiratory tract. In addition, with the exception of the Chilean H11 virus, little to no morbidity and absolutely no mortality was observed, leading to the conclusion that

further assessment of transmissibility in a ferret model was unnecessary. Nevertheless, experimental evidence provided by this study indicates that H11N9 viruses of South American origin are capable of replicating in the mammalian respiratory tract to some degree. In view of the previous studies in humans, the widespread presence of the H11 subtype and the lack of data on AIV infection in humans in South America (23,29) , further serological studies of poultry workers at LAMs in Colombia are necessary.

While the distribution and characteristics of AIV in North America, East Asia and Europe have been extensively studied, the prevalence and subtype diversity of AIV in South America remain understudied in spite of recent enhanced surveillance efforts(11,23,30,56–61). Unfortunately, this lack of knowledge continues to confound ecology of AIV in South America and its potential public health risk. Altogether, this study is significant because it suggests the H11N2 virus has the potential to establish itself in the Colombian poultry population, much like the Asian H9N2 subtype that was able to cross the domestic-species barrier and spread through LAMs in Bangladesh, China, Israel and South Korea, among others (18,62–64). However, these viruses may not need immediate intervention due to the lack of continued viral detection after the outbreak and reduced risk for mammalian infection. Overall, the paucity of data on AIV in LAMs in Colombia underscores how little is known about AIV ecology in South America, indicating the need for continued and increased active surveillance in this understudied continent.

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Author contribution:

Jimenez-Bluhm, P (self): Performed sample collection and screening. Performed viral isolation and sequencing. Performed phylogeny. Organized and constructed figures. Wrote manuscript.

Ciuoderis, KA Assisted in sample collection. Provided epidemiological data. Assisted in preparation of manuscript.

Cortez V Performed data analysis. Assisted in preparation of manuscript.

Hamilton-West, C Assisted in preparation of manuscript.

Karlsson, EA Performed *in vivo* and *in vitro* experiments. Organized and constructed figures. Edited and assisted greatly in the preparation of manuscript.

Osorio, JE Assisted in designing sampling strategies. Assisted in preparation of manuscript.

Schultz-Cherry, S Assisted in designing sampling strategies. Assisted in preparation and edition of manuscript.

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Table 1: Prevalence of influenza viruses at the LAM as determined by qrtPCR, by species

	No. screened	**No. positive**	**Percent positive**
Order *Anseriformes*	**419**	**10**	**2.3**
Domestic goose (*Anser anser domesticus*)	108	2	1.9
Domestic duck (*Anas platyrhynchos domesticus*)	311	8	2.6
Order *Galliformes*	**624**	**21**	**3.4**
Common quail (*Coturnix coturnix*)	5	0	
Indian peafowl (*Pavo cristatus*)	11	0	
Common pheasant (*Phasianus colchicus*)	22	0	
Helmeted guineafowl (*Numida meleagris*)	87	9	10.3
Japanese quail (*Coturnix japonica*)	89	3	3.4
Turkey (*Meleagris gallopavo*)	130	6	4.6
Domestic chicken (*Gallus gallus domesticus*)	280	3	1.1
Order *Columbiformes*	**15**	**0**	**0**
Rock dove (*Columba livia*)	15	0	
Unknown (Environmental)	102	0	**0**
Total	**1160**	**31**	**2.6**

Figure 1 Phylogenic trees of HA (**A**), NA (**B**) genes isolated from guineafowls at the LAM in Medellin, Colombia. Trees were generated using Maximum Likelihood method in MEGA software. Trees are based on full genetic sequence. Bootstrap values (n=500) greater than 70 indicated. Scale bars represent substitution per sites. Strains isolated in this study are indicated in black italics. Other strains characterized in this study are shown in black. AIV lineages are shown in color. Yellow, North American; Blue, South American, Green, Eurasian, Brown, short stalk NA.

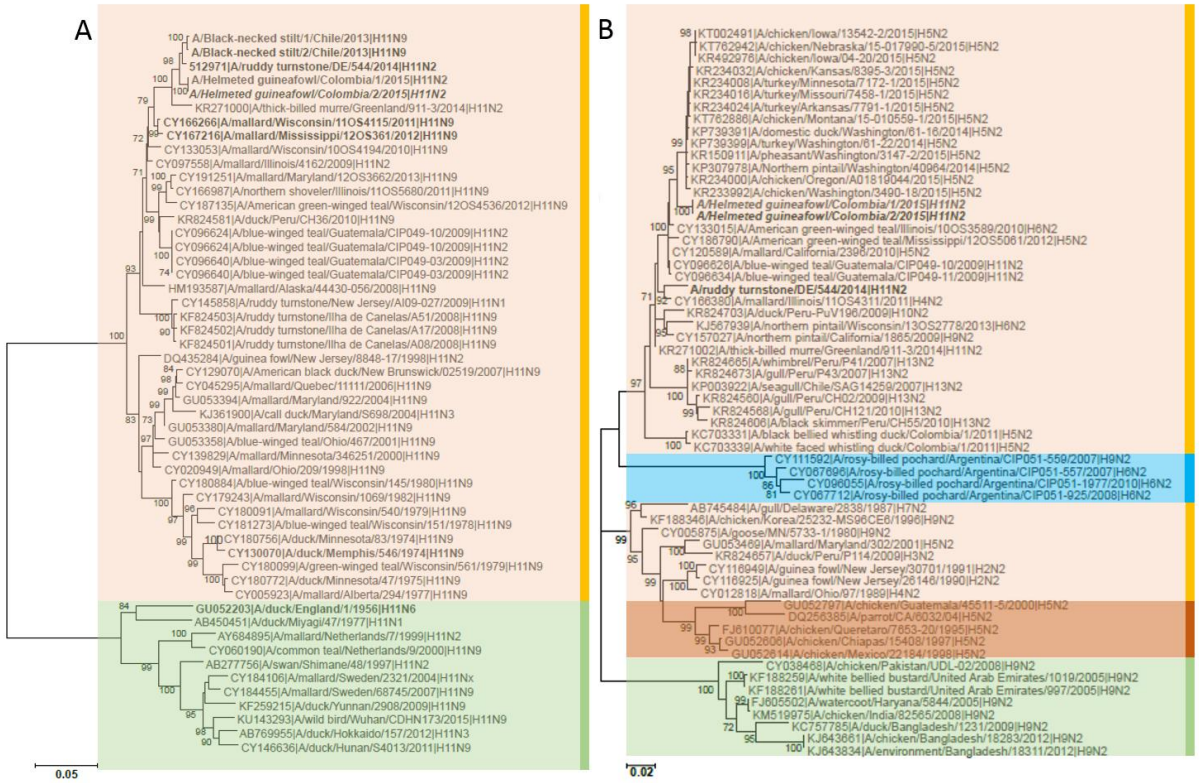

A
A/Black-necked stilt/1/Chile/2013|H11N9
A/Black-necked stilt/2/Chile/2013|H11N9
512971|A/ruddy turnstone/DE/544/2014|H11N2
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
KR271000|A/thick-billed murre/Greenland/911-3/2014|H11N2
CY166266|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY167216|A/mallard/Mississippi/12OS361/2012|H11N9
CY133053|A/mallard/Wisconsin/10OS4194/2010|H11N9
CY097558|A/mallard/Illinois/4162/2009|H11N2
CY191251|A/mallard/Maryland/12OS3662/2013|H11N9
CY166987|A/northern shoveler/Illinois/11OS5680/2011|H11N9
CY187135|A/American green-winged teal/Wisconsin/12OS4536/2012|H11N9
KR824581|A/duck/Peru/CH36/2010|H11N9
CY096624|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
CY096624|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
CY096640|A/blue-winged teal/Guatemala/CIP049-03/2009|H11N2
CY096640|A/blue-winged teal/Guatemala/CIP049-03/2009|H11N2
HM193587|A/mallard/Alaska/44430-056/2008|H11N9
CY145858|A/ruddy turnstone/New Jersey/AI09-027/2009|H11N1
KF824503|A/ruddy turnstone/Ilha de Canelas/A51/2008|H11N9
KF824502|A/ruddy turnstone/Ilha de Canelas/A17/2008|H11N9
KF824501|A/ruddy turnstone/Ilha de Canelas/A08/2008|H11N9
DQ435284|A/guinea fowl/New Jersey/8848-17/1998|H11N2
CY129070|A/American black duck/New Brunswick/02519/2007|H11N9
CY045295|A/mallard/Quebec/11111/2006|H11N9
GU053394|A/mallard/Maryland/922/2004|H11N9
KJ361900|A/call duck/Maryland/S698/2004|H11N3
GU053380|A/mallard/Maryland/584/2002|H11N9
GU053358|A/blue-winged teal/Ohio/467/2001|H11N9
CY139829|A/mallard/Minnesota/346251/2000|H11N9
CY020949|A/mallard/Ohio/209/1998|H11N9
CY180884|A/blue-winged teal/Wisconsin/145/1980|H11N9
CY179243|A/mallard/Wisconsin/1069/1982|H11N9
CY180091|A/mallard/Wisconsin/540/1979|H11N9
CY181273|A/blue-winged teal/Wisconsin/151/1978|H11N9
CY180756|A/duck/Minnesota/83/1974|H11N9
CY130070|A/duck/Memphis/546/1974|H11N9
CY180099|A/green-winged teal/Wisconsin/561/1979|H11N9
CY180772|A/duck/Minnesota/47/1975|H11N9
CY005923|A/mallard/Alberta/294/1977|H11N9
GU052203|A/duck/England/1/1956|H11N6
AB450451|A/duck/Miyagi/47/1977|H11N1
AY684895|A/mallard/Netherlands/7/1999|H11N2
CY060190|A/common teal/Netherlands/9/2000|H11N9
AB277756|A/swan/Shimane/48/1997|H11N2
CY184106|A/mallard/Sweden/2321/2004|H11Nx
CY184455|A/mallard/Sweden/68745/2007|H11N9
KF259215|A/duck/Yunnan/2908/2009|H11N9
KU143293|A/wild bird/Wuhan/CDHN173/2015|H11N9
AB769955|A/duck/Hokkaido/157/2012|H11N3
CY146636|A/duck/Hunan/S4013/2011|H11N9
0.05
B
KT002491|A/chicken/Iowa/13542-2/2015|H5N2
KT762942|A/chicken/Nebraska/15-017990-5/2015|H5N2
KR492976|A/chicken/Iowa/04-20/2015|H5N2
KR234032|A/chicken/Kansas/8395-3/2015|H5N2
KR234008|A/turkey/Minnesota/7172-1/2015|H5N2
KR234016|A/turkey/Missouri/7458-1/2015|H5N2
KR234024|A/turkey/Arkansas/7791-1/2015|H5N2
KT762886|A/chicken/Montana/15-010559-1/2015|H5N2
KP739391|A/domestic duck/Washington/61-16/2014|H5N2
KP739399|A/turkey/Washington/61-22/2014|H5N2
KR150911|A/pheasant/Washington/3147-2/2015|H5N2
KP307978|A/Northern pintail/Washington/40964/2014|H5N2
KR234000|A/chicken/Oregon/A01819044/2015|H5N2
KR233992|A/chicken/Washington/3490-18/2015|H5N2
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
CY133015|A/American green-winged teal/Illinois/10OS3589/2010|H6N2
CY186790|A/American green-winged teal/Mississippi/12OS5061/2012|H5N2
CY120589|A/mallard/California/2396/2010|H5N2
CY096626|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
CY096634|A/blue-winged teal/Guatemala/CIP049-11/2009|H11N2
A/ruddy turnstone/DE/544/2014|H11N2
CY166380|A/mallard/Illinois/11OS4311/2011|H4N2
KR824703|A/duck/Peru-PuV196/2009|H10N2
KJ567939|A/northern pintail/Wisconsin/13OS2778/2013|H6N2
CY157027|A/northern pintail/California/1865/2009|H9N2
KR271002|A/thick-billed murre/Greenland/911-3/2014|H11N2
KR824685|A/whimbrel/Peru/P41/2007|H13N2
KR824673|A/gull/Peru/P43/2007|H13N2
KP003922|A/seagull/Chile/SAG14259/2007|H13N2
KR824560|A/gull/Peru/CH02/2009|H13N2
KR824568|A/gull/Peru/CH121/2010|H13N2
KR824606|A/black skimmer/Peru/CH55/2010|H13N2
KC703331|A/black bellied whistling duck/Colombia/1/2011|H5N2
KC703339|A/white faced whistling duck/Colombia/1/2011|H5N2
CY111592|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
CY067696|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
CY096055|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY067712|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
AB745484|A/gull/Delaware/2838/1987|H7N2
KF188346|A/chicken/Korea/25232-MS96CE6/1996|H9N2
CY005875|A/goose/MN/5733-1/1980|H9N2
GU053469|A/mallard/Maryland/302/2001|H5N2
KR824657|A/duck/Peru/P114/2009|H3N2
CY116949|A/guinea fowl/New Jersey/30701/1991|H2N2
CY116925|A/guinea fowl/New Jersey/26146/1990|H2N2
CY012818|A/mallard/Ohio/97/1989|H4N2
GU052797|A/chicken/Guatemala/45511-5/2000|H5N2
DQ256385|A/parrot/CA/6032/04|H5N2
FJ610077|A/chicken/Queretaro/7653-20/1995|H5N2
GU052806|A/chicken/Chiapas/15408/1997|H5N2
GU052614|A/chicken/Mexico/22184/1998|H5N2
CY038468|A/chicken/Pakistan/UDL-02/2008|H9N2
KF188259|A/white bellied bustard/United Arab Emirates/1019/2005|H9N2
KF188261|A/white bellied bustard/United Arab Emirates/997/2005|H9N2
FJ605502|A/watercoot/Haryana/5844/2005|H9N2
KM519975|A/chicken/India/82565/2008|H9N2
KC757785|A/duck/Bangladesh/1231/2009|H9N2
KJ643861|A/chicken/Bangladesh/18283/2012|H9N2
KJ643834|A/environment/Bangladesh/18311/2012|H9N2
0.02

Table 2: Influenza viruses most closely related to the Colombian H11N2 viruses, as established by BLAST. Nucleotide identity indicated in percentage.

	Most closely related genes segment					
H11 virus	**PB2**	**PB1**	**PA**	**NP**	**M**	**NS**
A/helmeted guineafowl/Colombia/ 1/2015	A/white-winged scoter/ Wisconsin/ 10OS3922/ 2010 H14N8 99%	A/mallard/Maryland/ 14OS1447/ 2014 H3N9 99%	A/mallard/Ohio/ 11OS2229/ 2011 H5N2 99%	A/mallard/Alberta/ 243/2006 H7N3 98%	A/blue-winged teal/Texas/AI12-3566/2012 H4N6 99%	A/blue-winged teal/Iowa/ 13OS2349/2013 H4N8 99%
A/helmeted guineafowl/Colombia/ 2/2015	A/white-winged scoter/ Wisconsin /10OS3922/ 2010 H14N8 99%	A/mallard/Maryland/ 14OS1447/ 2014 H3N9 99%	A/mallard/Ohio/ 11OS2229/ 2011 H5N2 99%	A/mallard/Alberta/ 243/2006 H7N3 98%	A/blue-winged teal/Texas/AI12-3566/2012 H4N6 99%	A/blue-winged teal/Iowa/ 13OS2349/2013 H4N8 99%

Figure 2 Phylogenic trees of the HA gene. Strains sequenced in this study are indicated in black italics. Trees were generated using Maximum Likelihood method in MEGA software. Trees are based on partial sequence. Bootstrap values (n=500) greater than 70 indicated. Scale bars represent substitution per sites. Yellow, North American; Blue, South American; Green, Eurasian.

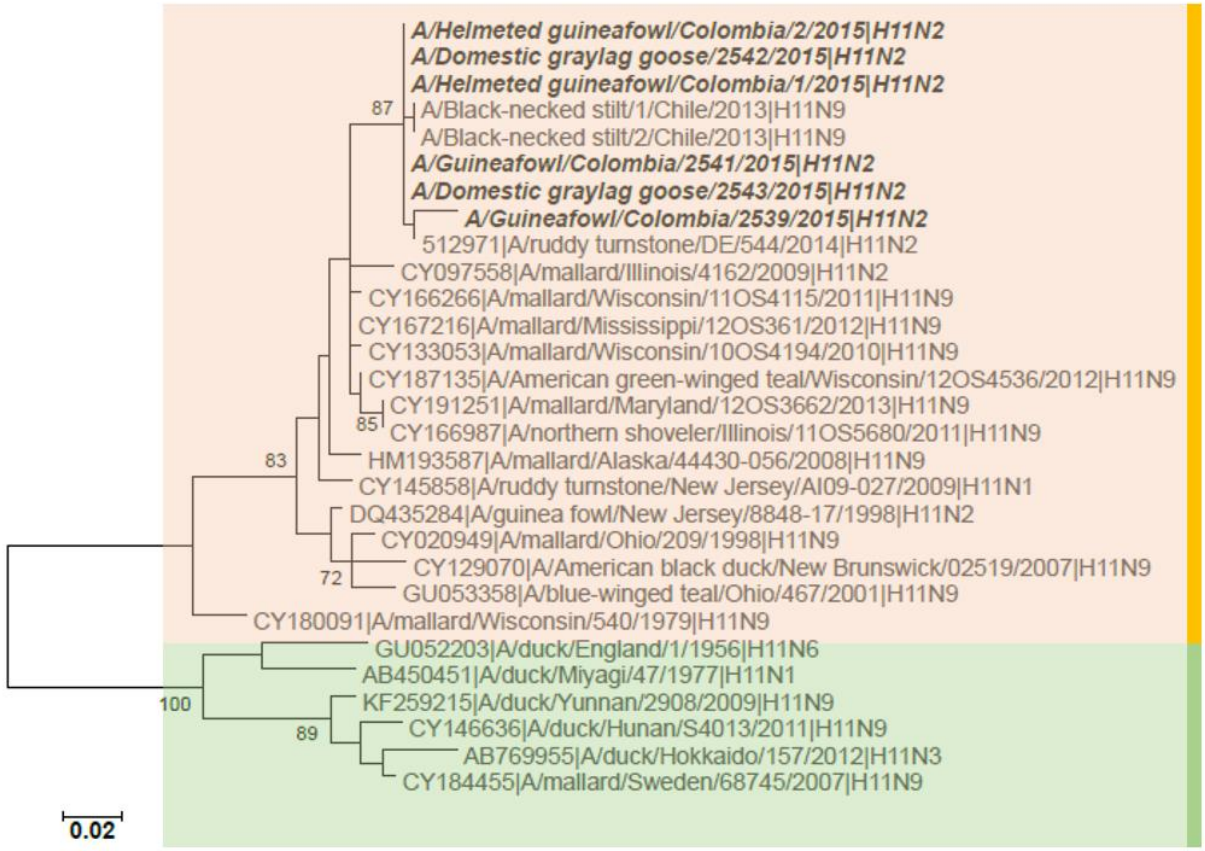

A/Helmeted guineafowl/Colombia/2/2015|H11N2
A/Domestic graylag goose/2542/2015|H11N2
A/Helmeted guineafowl/Colombia/1/2015|H11N2
87
A/Black-necked stilt/1/Chile/2013|H11N9
A/Black-necked stilt/2/Chile/2013|H11N9
A/Guineafowl/Colombia/2541/2015|H11N2
A/Domestic graylag goose/2543/2015|H11N2
A/Guineafowl/Colombia/2539/2015|H11N2
512971|A/ruddy turnstone/DE/544/2014|H11N2
CY097558|A/mallard/Illinois/4162/2009|H11N2
CY166266|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY167216|A/mallard/Mississippi/12OS361/2012|H11N9
CY133053|A/mallard/Wisconsin/10OS4194/2010|H11N9
CY187135|A/American green-winged teal/Wisconsin/12OS4536/2012|H11N9
CY191251|A/mallard/Maryland/12OS3662/2013|H11N9
85
CY166987|A/northern shoveler/Illinois/11OS5680/2011|H11N9
83
HM193587|A/mallard/Alaska/44430-056/2008|H11N9
CY145858|A/ruddy turnstone/New Jersey/AI09-027/2009|H11N1
DQ435284|A/guinea fowl/New Jersey/8848-17/1998|H11N2
CY020949|A/mallard/Ohio/209/1998|H11N9
72
CY129070|A/American black duck/New Brunswick/02519/2007|H11N9
GU053358|A/blue-winged teal/Ohio/467/2001|H11N9
CY180091|A/mallard/Wisconsin/540/1979|H11N9
GU052203|A/duck/England/1/1956|H11N6
AB450451|A/duck/Miyagi/47/1977|H11N1
100
KF259215|A/duck/Yunnan/2908/2009|H11N9
89
CY146636|A/duck/Hunan/S4013/2011|H11N9
AB769955|A/duck/Hokkaido/157/2012|H11N3
CY184455|A/mallard/Sweden/68745/2007|H11N9
0.02

Figure 3 Characterization of the receptor binding properties of isolated H11 viruses. The viruses were tested for their ability to bind to α2,3 and α2,6 sialyglycopolymers. The figure shows absorbency of the wells, versus concentration of the polymer. Error bars represent the standard error of the mean.

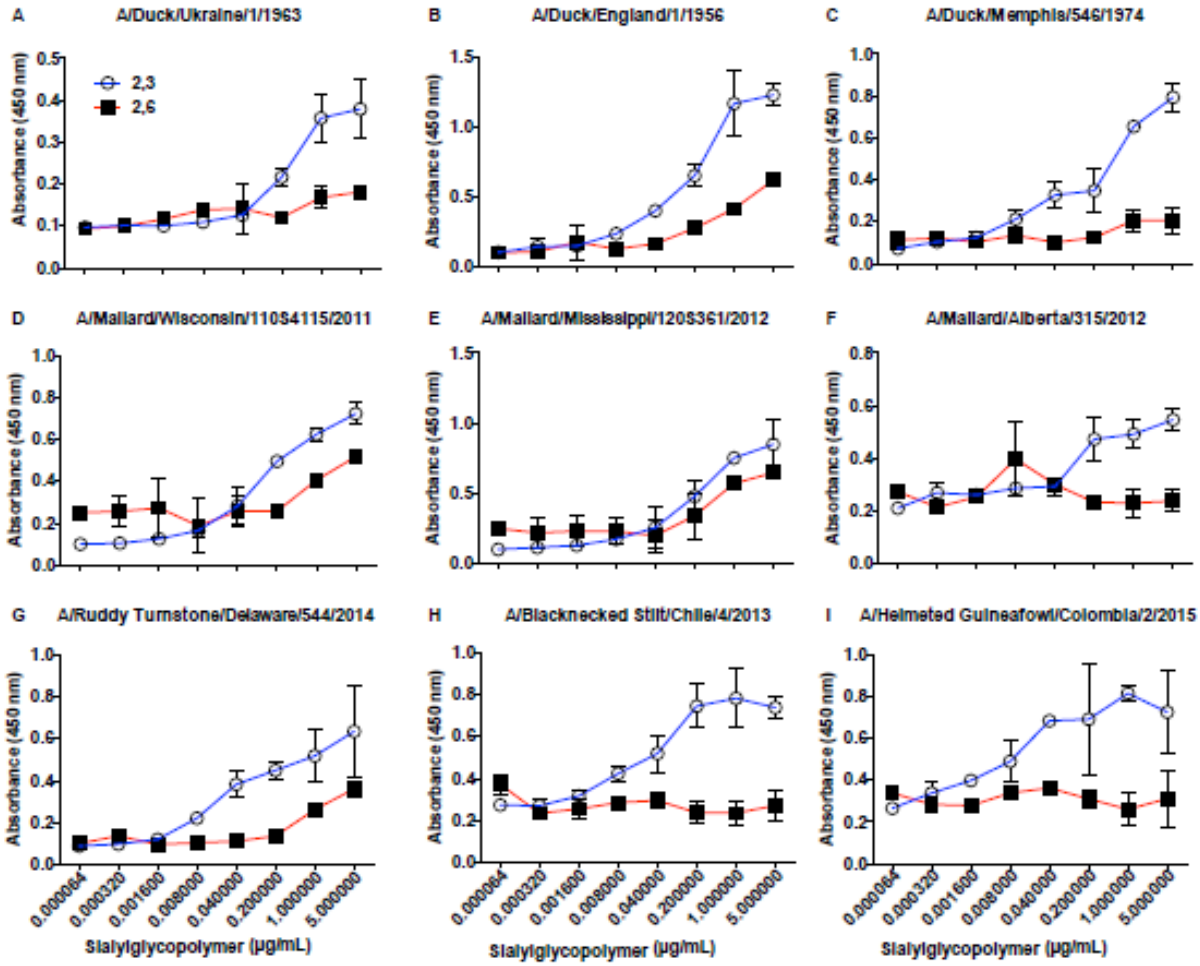

A
A/Duck/Ukraine/1/1963
2,3
2,6
B
A/Duck/England/1/1956
C
A/Duck/Memphis/546/1974
D
A/Mallard/Wisconsin/11084115/2011
E
A/Mallard/Mississippi/12083361/2012
F
A/Mallard/Alberta/315/2012
G
A/Ruddy Turnstone/Delaware/544/2014
H
A/Blacknecked Stilt/Chile/4/2013
I
A/Helmeted Guineafowl/Colombia/2/2015
Absorbance (450 nm)
0.000064
0.000320
0.001600
0.008000
0.040000
0.200000
1.000000
5.000000
Sialylglycopolymer (µg/mL)

Table 3. Growth and transmission of Colombian H11N2 viruses in chickens

	Virus - Cloacal - Titers (EID_{50}[1])							
	Duck/Mem		**RT/DE**		**BNS/Chile**		**HGF/Colombia**	
dpi	**Direct**	**Contact**	**Direct**	**Contact**	**Direct**	**Contact**	**Direct**	**Contact**
0	n.d.[2] (0%)[3]	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
2	4.0-5.0 (100%)	n.d. (0%)	3.5-4.5 (60%)	n.d. (0%)	5.0-6.5 (100%)	n.d. (0%)	4.5-6.5 (100%)	n.d. (0%)
4	4.5-6.0 (100%)	2.5 (20%)	3.5-4.5 (60%)	2.5 (20%)	4.5-5.5 (80%)	n.d. (0%)	4.5-6.5 (100%)	4.5-5.5 (60%)
6	3.5-4.0 (40%)	n.d. (0%)	2.5-3.5 (40%)	n.d. (0%)	2.5-4.5 (80%)	n.d. (0%)	3.5-5.5 (80%)	2.5-4.5 (60%)
8	2.5 (20%)	n.d. (0%)	2.5 (20%)	n.d. (0%)	2.5 (20%)	n.d. (0%)	2.5 (40%)	2.5 (20%)
10	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	2.5 (20%)	n.d. (0%)
12	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
14	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
	Virus - Oropharyngeal - Titers (EID_{50})							
	Duck/Mem		**RT/DE**		**BNS/Chile**		**HGF/Colombia**	
dpi	**Direct**	**Contact**	**Direct**	**Contact**	**Direct**	**Contact**	**Direct**	**Contact**
0	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
2	2.5-4.0 (100%)	n.d. (0%)	3.0-4.0 (40%)	n.d. (0%)	2.5-4.5 (100%)	n.d. (0%)	3.0-4.5 (100%)	n.d. (0%)
4	2.5-3.0 (100%)	n.d (0%)	2.5-3.0 (60%)	n.d (0%)	2.5 (60%)	n.d. (0%)	2.5-3.5 (100%)	n.d. (0%)
6	n.d (0%)	n.d. (0%)	2.5 (20%)	n.d. (0%)	n.d (0%)	n.d. (0%)	2.5 (40%)	n.d. (0%)
8	n.d (0%)	n.d. (0%)	n.d (0%)	n.d. (0%)	n.d (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
10	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
12	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)
14	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%)	n.d. (0%).	n.d. (0%)

Abbreviations: dpi, days post-infection;

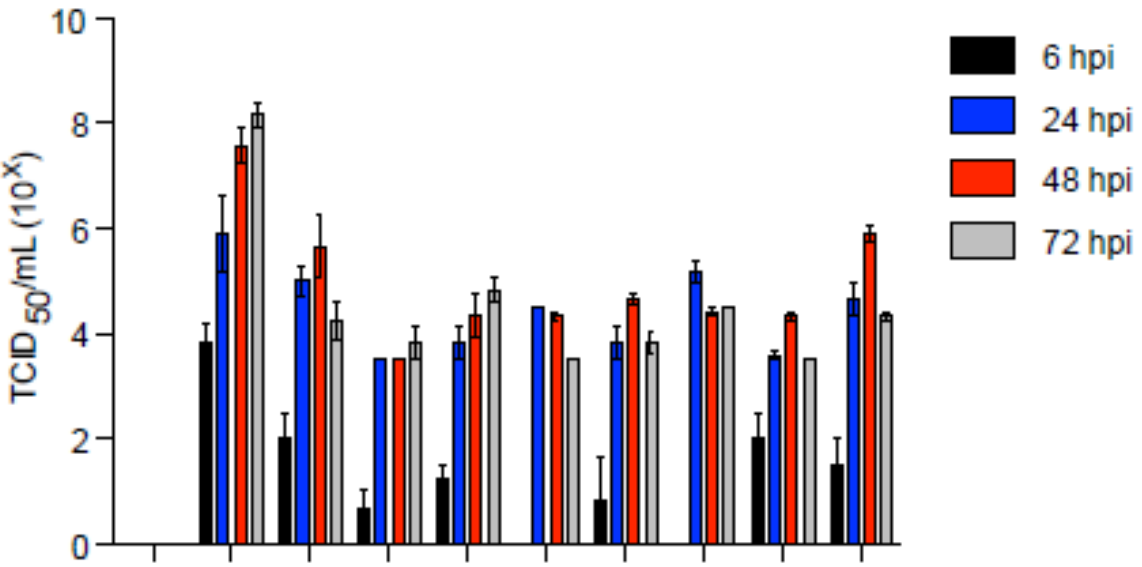
[1] log10 EID_{50}/ml. Data are the average of 5 animals/group.

[2] n.d.: not detected. Values were below the limit of detection (<1 $\log_{10}$ EID_{50}/100 µL)

[3] parenthesis indicates the percentage of animals shedding

Figure 4 Replication of H11N2 and H11N9 viruses in vitro. **(A)** MDCK and **(B)** A549 cells were infected at a MOI of 0.01 and supernatants were titrated as $TDIC_{50}$ at 6, 24, 48 and 72 hpi. Error bars represent the standard error of the mean.

A


10
8
6
4
2
0
TCID50/mL (10X)
6 hpi
24 hpi
48 hpi
72 hpi

B

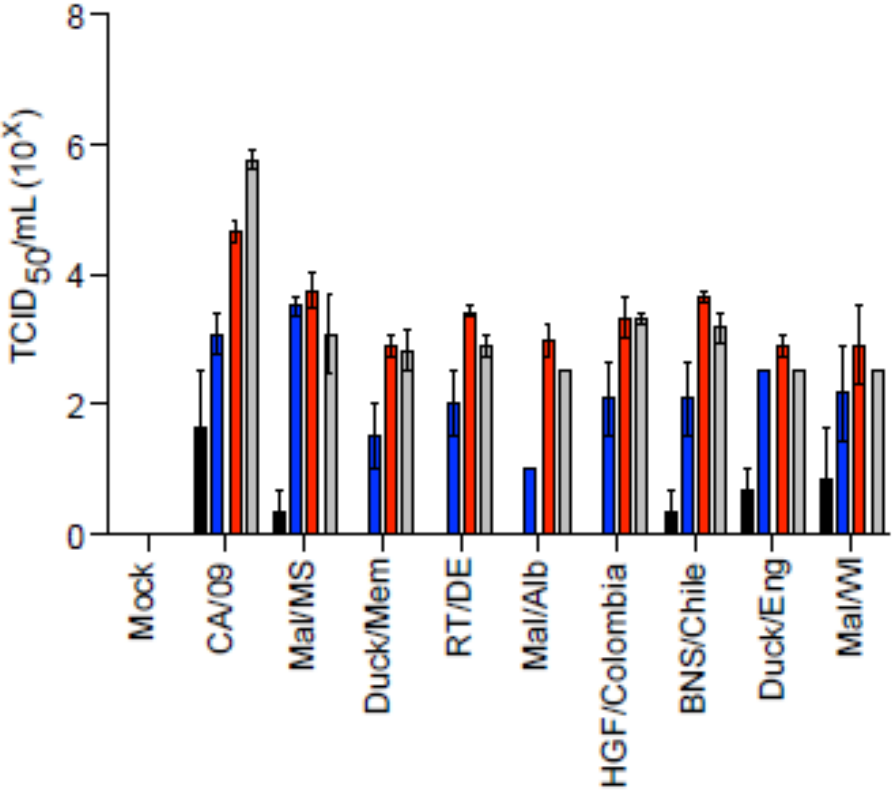
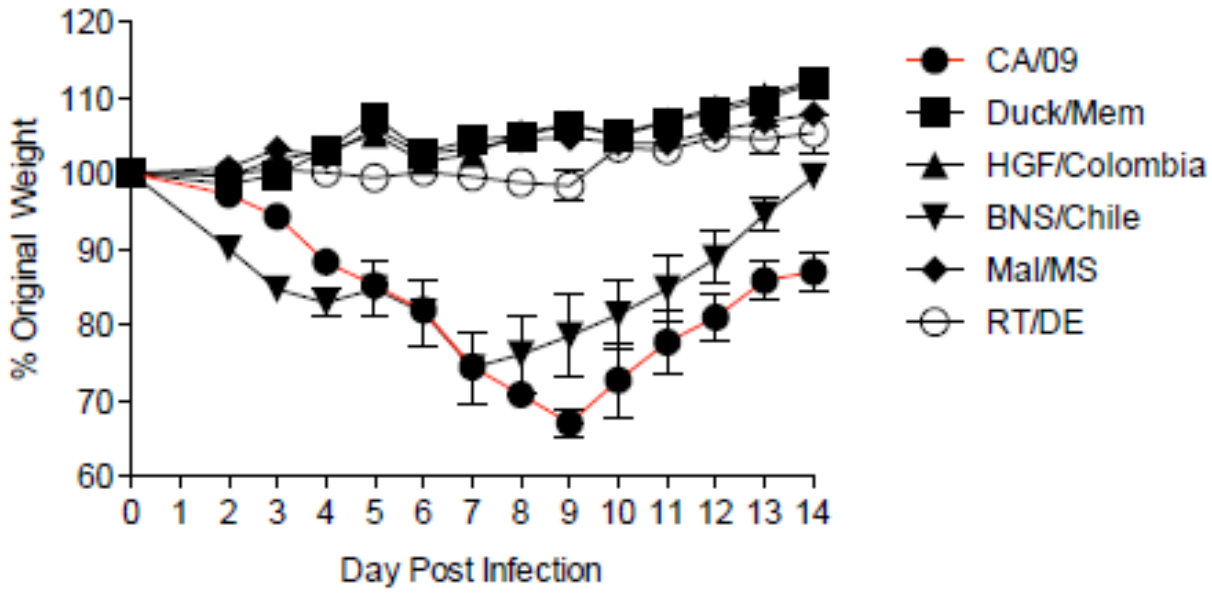
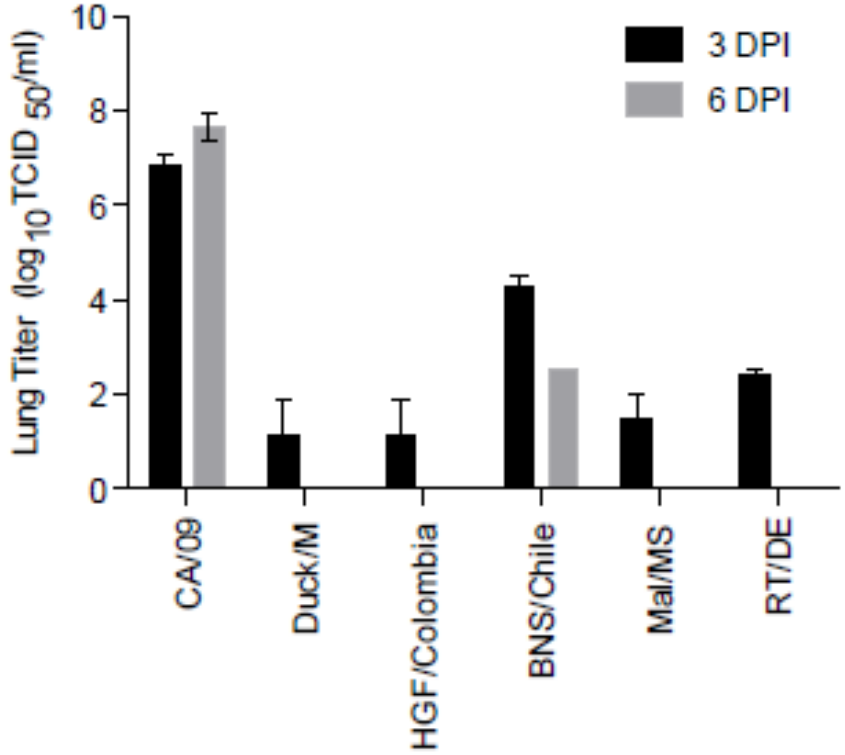

8
6
4
2
0
TCID50/mL (10X)
Mock
CA/09
Mal/MS
Duck/Mem
RT/DE
Mal/Alb
HGF/Colombia
BNS/Chile
Duck/Eng
Mal/WI

Figure 5 Pathogenicity of H11 viruses in vivo. 6 to 8 week old female Balb/c mice (n=11) where intranasally infected with 104 TCID50 of challenge and control viruses. **(A)** Weight loss was monitored for 14 dpi and **(B)** 3 and 6 dpi, lungs were collected from 3 mice per virus strain and homogenates were tittered in $TCID_{50}$.

A


120
110
100
90
80
70
60
% Original Weight
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Day Post Infection
CA/09
Duck/Mem
HGF/Colombia
BNS/Chile
Mal/MS
RT/DE

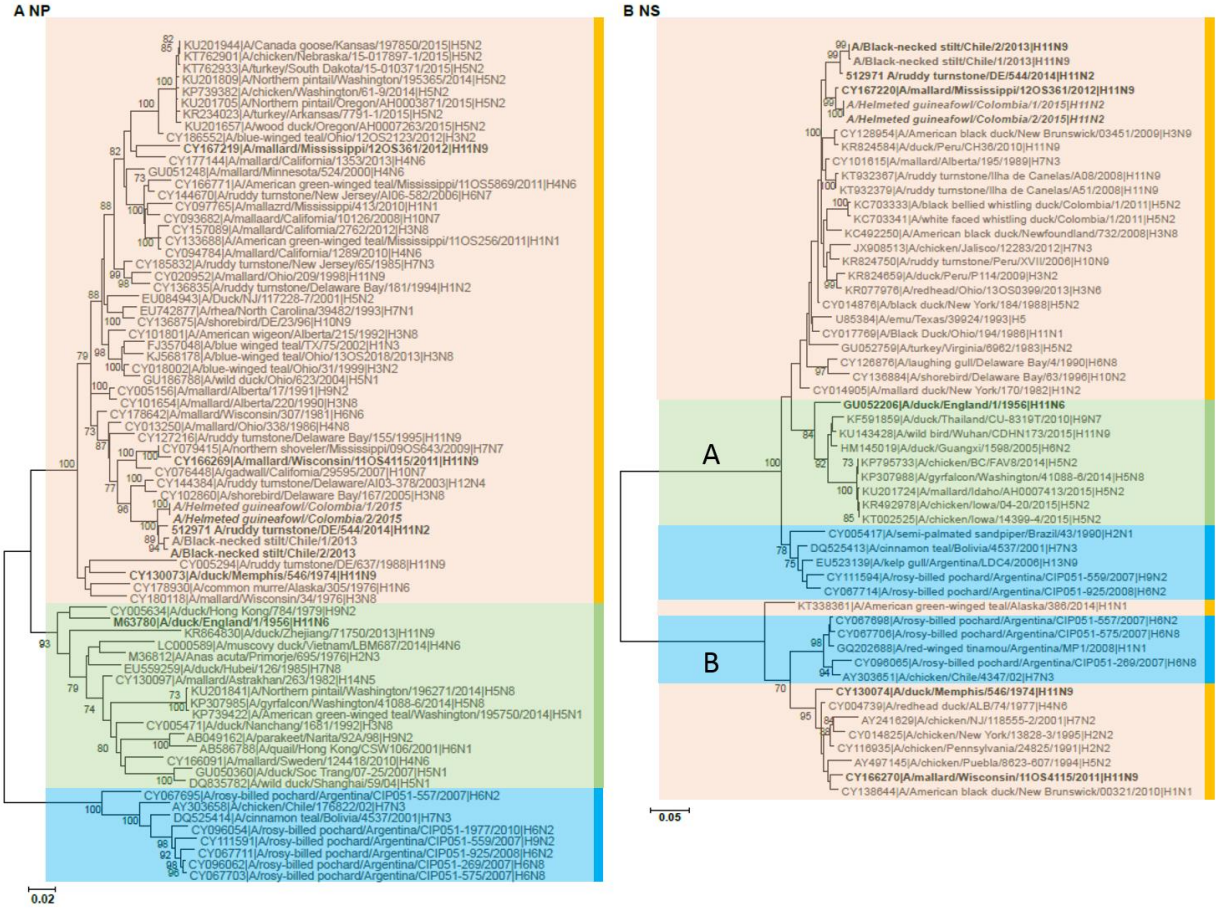
B


10
8
6
4
2
0
Lung Titer (log 10 TCID 50/ml)
3 DPI
6 DPI
CA/09
Duck/M
HGF/Colombia
BNS/Chile
Mal/MS
RT/DE

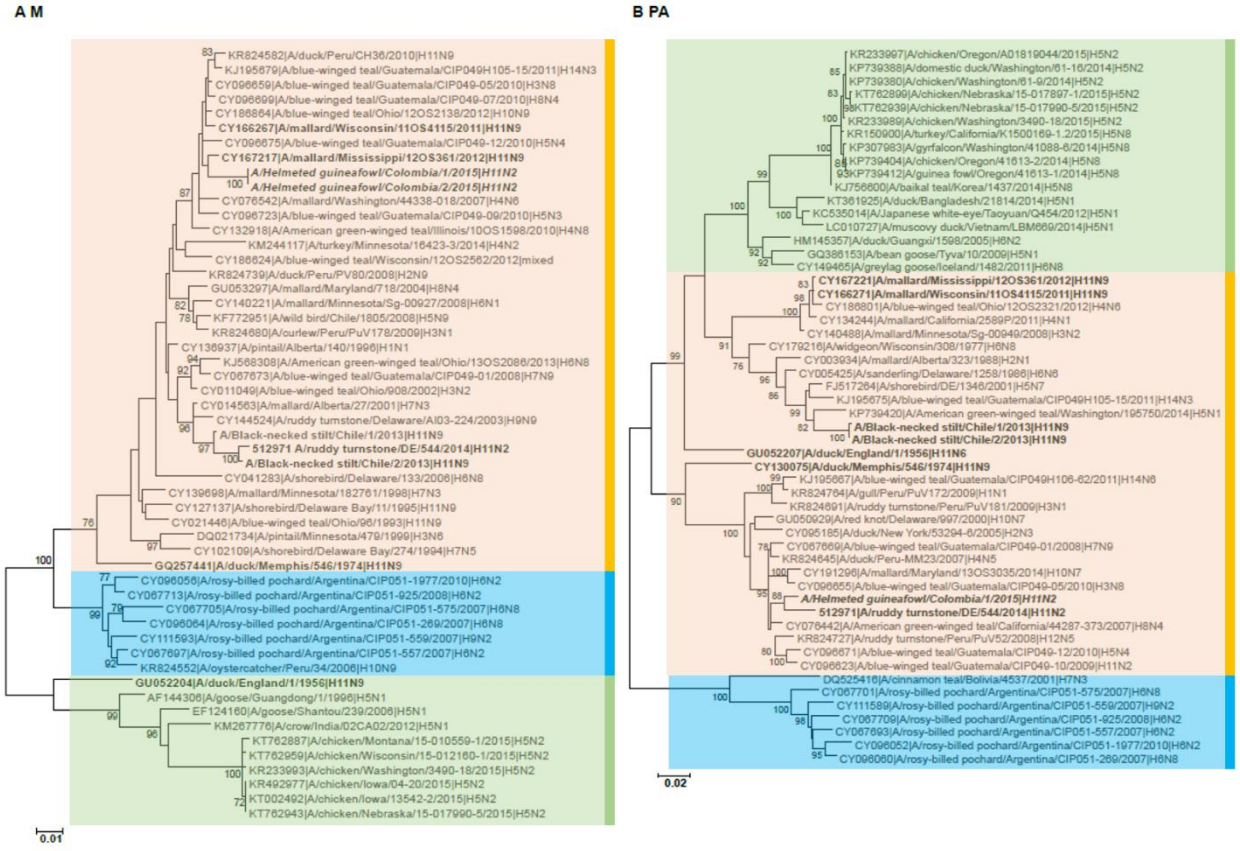
Supplementary Table S1 Nucleotide (NT) and amino acid (AA) similarity (%) between HGF/Colombia and BNS/Chile, by gene segment. N/A, not applicable.

	Segment								
Viruses	**PB2**	**PB1**	**PA**	**HA**	**NP**	**NA**	**MP**	**NS**	
HGF/Colombia (H11N2)	97	97	87	98	98	N/A	96	95	**NT**
BNS/Chile (H11N9)	99	99	98	98	99	N/A	99	93	**AA**

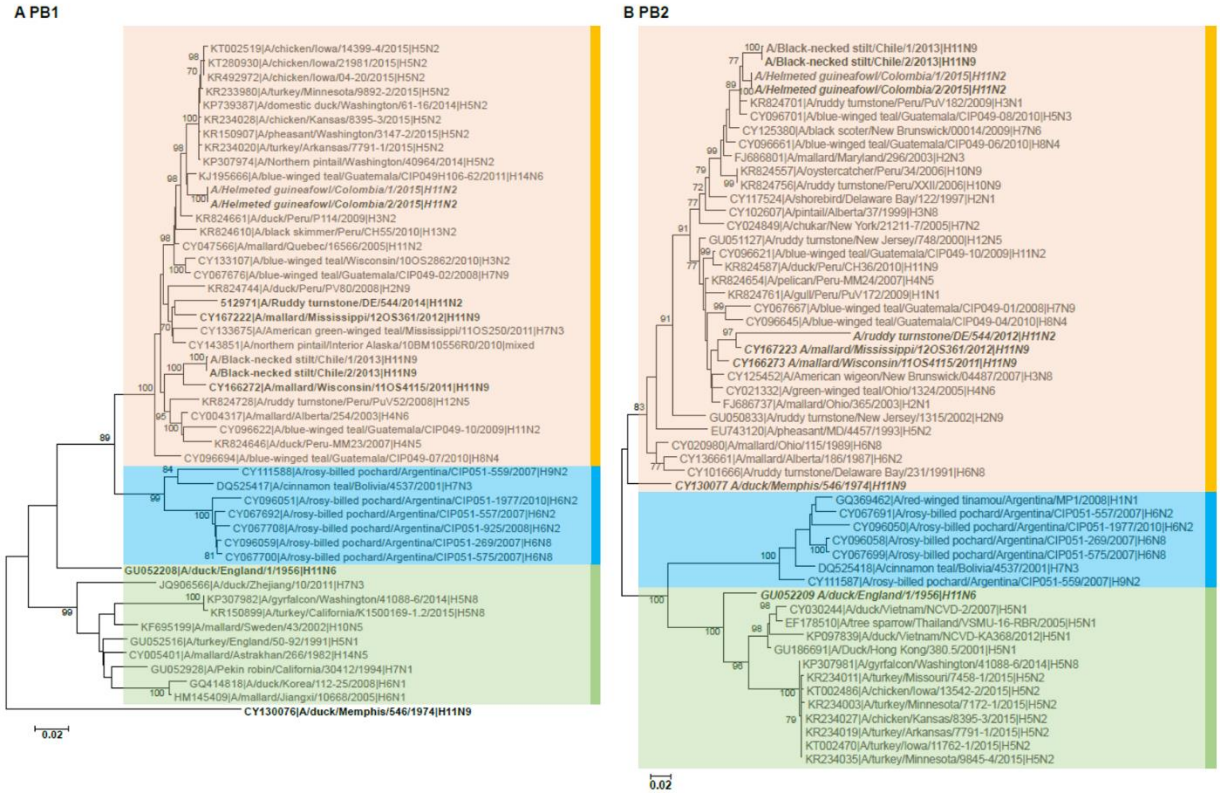
Supplementary Figure S1 Phylogenic trees of NP (**A**), NS (**B**). Strains isolated in this study are indicated in black italics. Other strains characterized in this study are shown in black. AIV lineages are shown in color. Yellow, North American; Blue, South American; Green, Eurasian.


A NP
KU201944|A/Canada goose/Kansas/197850/2015|H5N2
KT762901|A/chicken/Nebraska/15-017897-1/2015|H5N2
KT762933|A/turkey/South Dakota/15-010371/2015|H5N2
KU201809|A/Northern pintail/Washington/195365/2014|H5N2
KP739382|A/chicken/Washington/61-9/2014|H5N2
KU201705|A/Northern pintail/Oregon/AH0003871/2015|H5N2
KR234023|A/turkey/Arkansas/7791-1/2015|H5N2
KU201657|A/wood duck/Oregon/AH0007263/2015|H5N2
CY186552|A/blue-winged teal/Ohio/12OS2123/2012|H3N2
CY167219|A/mallard/Mississippi/12OS361/2012|H11N9
CY177144|A/mallard/California/1353/2013|H4N6
GU051248|A/mallard/Minnesota/524/2000|H4N6
CY166771|A/American green-winged teal/Mississippi/11OS5869/2011|H4N6
CY144670|A/ruddy turnstone/New Jersey/AI06-582/2006|H6N7
CY097765|A/mallazrd/Mississippi/413/2010|H1N1
CY093682|A/mallaard/California/10126/2008|H10N7
CY157089|A/mallard/California/2762/2012|H3N8
CY133688|A/American green-winged teal/Mississippi/11OS256/2011|H1N1
CY094784|A/mallard/California/1289/2010|H4N6
CY185832|A/ruddy turnstone/New Jersey/65/1985|H7N3
CY020952|A/mallard/Ohio/209/1998|H11N9
CY136835|A/ruddy turnstone/Delaware Bay/181/1994|H1N2
EU084943|A/Duck/NJ/117228-7/2001|H5N2
EU742877|A/rhea/North Carolina/39482/1993|H7N1
CY136875|A/shorebird/DE/23/96|H10N9
CY101801|A/American wigeon/Alberta/215/1992|H3N8
FJ357048|A/blue winged teal/TX/752002|H1N3
KJ568178|A/blue-winged teal/Ohio/13OS2018/2013|H3N8
CY018002|A/blue-winged teal/Ohio/31/1999|H3N2
GU186788|A/wild duck/Ohio/623/2004|H5N1
CY005156|A/mallard/Alberta/17/1991|H9N2
CY101654|A/mallard/Alberta/220/1990|H3N8
CY178642|A/mallard/Wisconsin/307/1981|H6N6
CY013250|A/mallard/Ohio/338/1986|H4N8
CY127216|A/ruddy turnstone/Delaware Bay/155/1995|H11N9
CY079415|A/northern shoveler/Mississippi/09OS643/2009|H7N7
CY166269|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY076448|A/gadwall/California/29595/2007|H10N7
CY144384|A/ruddy turnstone/Delaware/AI03-378/2003|H12N4
CY102860|A/shorebird/Delaware Bay/167/2005|H3N8
A/Helmeted guineafowl/Colombia/1/2015
A/Helmeted guineafowl/Colombia/2/2015
512971 A/ruddy turnstone/DE/544/2014|H11N2
A/Black-necked stilt/Chile/1/2013
A/Black-necked stilt/Chile/2/2013
CY005294|A/ruddy turnstone/DE/637/1988|H11N9
CY130073|A/duck/Memphis/546/1974|H11N9
CY178930|A/common murre/Alaska/305/1976|H1N6
CY180118|A/mallard/Wisconsin/34/1976|H3N8
CY005634|A/duck/Hong Kong/784/1979|H9N2
M63780|A/duck/England/1/1956|H11N6
KR864830|A/duck/Zhejiang/71750/2013|H11N9
LC000589|A/muscovy duck/Vietnam/LBM687/2014|H4N6
M36812|A/Anas acuta/Primorje/695/1976|H2N3
EU559259|A/duck/Hubei/126/1965|H7N8
CY130097|A/mallard/Astrakhan/263/1982|H14N5
KU201841|A/Northern pintail/Washington/196271/2014|H5N8
KP307985|A/gyrfalcon/Washington/41088-6/2014|H5N8
KP739422|A/American green-winged teal/Washington/195750/2014|H5N1
CY005471|A/duck/Nanchang/1681/1992|H3N8
AB049162|A/parakeet/Narita/92A/98|H9N2
AB586786|A/quail/Hong Kong/CSW106/2001|H6N1
CY166091|A/mallard/Sweden/124418/2010|H4N6
GU050360|A/duck/Soc Trang/07-25/2007|H5N1
DQ835782|A/wild duck/Shanghai/59/04|H5N1
CY067695|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
AY303658|A/chicken/Chile/176822/02|H7N3
DQ525414|A/cinnamon teal/Bolivia/4537/2001|H7N3
CY096054|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY111591|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
CY067711|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
CY096062|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
CY067703|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
0.02
B NS
A/Black-necked stilt/Chile/2/2013|H11N9
A/Black-necked stilt/Chile/1/2013|H11N9
512971 A/ruddy turnstone/DE/544/2014|H11N2
CY167220|A/mallard/Mississippi/12OS361/2012|H11N9
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
CY128954|A/American black duck/New Brunswick/03451/2009|H3N9
KR824584|A/duck/Peru/CH36/2010|H11N9
CY101615|A/mallard/Alberta/195/1989|H7N3
KT932367|A/ruddy turnstone/Ilha de Canelas/A08/2008|H11N9
KT932379|A/ruddy turnstone/Ilha de Canelas/A51/2008|H11N9
KC703333|A/black bellied whistling duck/Colombia/1/2011|H5N2
KC703341|A/white faced whistling duck/Colombia/1/2011|H5N2
KC492250|A/American black duck/Newfoundland/732/2008|H3N8
JX908513|A/chicken/Jalisco/12283/2012|H7N3
KR824750|A/ruddy turnstone/Peru/XVII/2006|H10N9
KR824659|A/duck/Peru/P114/2009|H3N2
KR077976|A/redhead/Ohio/13OS0399/2013|H3N6
CY014876|A/black duck/New York/184/1988|H5N2
U85384|A/emu/Texas/39924/1993|H5
CY017769|A/Black Duck/Ohio/194/1986|H11N1
GU052759|A/turkey/Virginia/6962/1983|H5N2
CY126876|A/laughing gull/Delaware Bay/4/1990|H6N8
CY136884|A/shorebird/Delaware Bay/63/1996|H10N2
CY014905|A/mallard duck/New York/170/1982|H1N2
GU052206|A/duck/England/1/1956|H11N6
KF591859|A/duck/Thailand/CU-8319T/2010|H9N7
KU143428|A/wild bird/Wuhan/CDHN173/2015|H11N9
HM145019|A/duck/Guangxi/1598/2005|H6N2
KP795733|A/chicken/BC/FAV8/2014|H5N2
KP307988|A/gyrfalcon/Washington/41088-6/2014|H5N8
KU201724|A/mallard/Idaho/AH0007413/2015|H5N2
KR492978|A/chicken/Iowa/04-20/2015|H5N2
KT002525|A/chicken/Iowa/14399-4/2015|H5N2
A
CY005417|A/semi-palmated sandpiper/Brazil/43/1990|H2N1
DQ525413|A/cinnamon teal/Bolivia/4537/2001|H7N3
EU523139|A/kelp gull/Argentina/LDC4/2006|H13N9
CY111594|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
CY067714|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
KT338361|A/American green-winged teal/Alaska/386/2014|H1N1
CY067698|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
CY067706|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
GQ202688|A/red-winged tinamou/Argentina/MP1/2008|H1N1
CY096065|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
AY303651|A/chicken/Chile/4347/02|H7N3
B
CY130074|A/duck/Memphis/546/1974|H11N9
CY004739|A/redhead duck/ALB/74/1977|H4N6
AY241629|A/chicken/NJ/118555-2/2001|H7N2
CY014825|A/chicken/New York/13828-3/1995|H2N2
CY116935|A/chicken/Pennsylvania/24825/1991|H2N2
AY497145|A/chicken/Puebla/8623-607/1994|H5N2
CY166270|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY138644|A/American black duck/New Brunswick/00321/2010|H1N1
0.05

Supplementary Figure S2 Phylogenic trees of M (**A**), PA (**B**). Strains isolated in this study are indicated in black italics. Other strains characterized in this study are shown in black. AIV lineages are shown in color. Yellow, North American; Blue, South American; Green, Eurasian.


A M
KR824582|A/duck/Peru/CH36/2010|H11N9
KJ195679|A/blue-winged teal/Guatemala/CIP049H105-15/2011|H14N3
CY096659|A/blue-winged teal/Guatemala/CIP049-05/2010|H3N8
CY096699|A/blue-winged teal/Guatemala/CIP049-07/2010|H8N4
CY186864|A/blue-winged teal/Ohio/12OS2138/2012|H10N9
CY166267|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY096675|A/blue-winged teal/Guatemala/CIP049-12/2010|H5N4
CY167217|A/mallard/Mississippi/12OS361/2012|H11N9
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
CY076542|A/mallard/Washington/44338-018/2007|H4N6
CY096723|A/blue-winged teal/Guatemala/CIP049-09/2010|H5N3
CY132918|A/American green-winged teal/Illinois/10OS1598/2010|H4N8
KM244117|A/turkey/Minnesota/16423-3/2014|H4N2
CY186624|A/blue-winged teal/Wisconsin/12OS2562/2012|mixed
KR824739|A/duck/Peru/PV80/2008|H2N9
GU053297|A/mallard/Maryland/718/2004|H8N4
CY140221|A/mallard/Minnesota/Sg-00927/2008|H6N1
KF772951|A/wild bird/Chile/1805/2008|H5N9
KR824680|A/curlew/Peru/PuV178/2009|H3N1
CY136937|A/pintail/Alberta/140/1996|H1N1
KJ568308|A/American green-winged teal/Ohio/13OS2086/2013|H6N8
CY067673|A/blue-winged teal/Guatemala/CIP049-01/2008|H7N9
CY011049|A/blue-winged teal/Ohio/908/2002|H3N2
CY014563|A/mallard/Alberta/27/2001|H7N3
CY144524|A/ruddy turnstone/Delaware/AI03-224/2003|H9N9
A/Black-necked stilt/Chile/1/2013|H11N9
512971 A/ruddy turnstone/DE/544/2014|H11N2
A/Black-necked stilt/Chile/2/2013|H11N9
CY041283|A/shorebird/Delaware/133/2006|H8N8
CY139698|A/mallard/Minnesota/182761/1998|H7N3
CY127137|A/shorebird/Delaware Bay/11/1995|H11N9
CY021446|A/blue-winged teal/Ohio/96/1993|H11N9
DQ021734|A/pintail/Minnesota/479/1999|H3N8
CY102109|A/shorebird/Delaware Bay/274/1994|H7N5
GQ257441|A/duck/Memphis/546/1974|H11N9
CY096056|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY067713|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
CY067705|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
CY096064|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
CY111593|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
CY067697|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
KR824552|A/oystercatcher/Peru/34/2006|H10N9
GU052204|A/duck/England/1/1956|H11N9
AF144306|A/goose/Guangdong/1/1996|H5N1
EF124160|A/goose/Shantou/239/2006|H5N1
KM267776|A/crow/India/02CA02/2012|H5N1
KT762887|A/chicken/Montana/15-010559-1/2015|H5N2
KT762959|A/chicken/Wisconsin/15-012160-1/2015|H5N2
KR233993|A/chicken/Washington/3490-18/2015|H5N2
KR492977|A/chicken/Iowa/04-20/2015|H5N2
KT002492|A/chicken/Iowa/13542-2/2015|H5N2
KT762943|A/chicken/Nebraska/15-017990-5/2015|H5N2
0.01
B PA
KR233997|A/chicken/Oregon/A01819044/2015|H5N2
KP739388|A/domestic duck/Washington/61-16/2014|H5N2
KP739380|A/chicken/Washington/61-9/2014|H5N2
KT762899|A/chicken/Nebraska/15-017897-1/2015|H5N2
KT762939|A/chicken/Nebraska/15-017990-5/2015|H5N2
KR233989|A/chicken/Washington/3490-18/2015|H5N2
KR150900|A/turkey/California/K1500169-1.2/2015|H5N8
KP307983|A/gyrfalcon/Washington/41088-6/2014|H5N8
KP739404|A/chicken/Oregon/41613-2/2014|H5N8
KP739412|A/guinea fowl/Oregon/41613-1/2014|H5N8
KJ756600|A/baikal teal/Korea/1437/2014|H5N8
KT361925|A/duck/Bangladesh/21814/2014|H5N1
KC535014|A/Japanese white-eye/Taoyuan/Q454/2012|H5N1
LC010727|A/muscovy duck/Vietnam/LBM866/2014|H5N1
HM145357|A/duck/Guangxi/1598/2005|H8N2
GQ386153|A/bean goose/Tyva/10/2009|H5N1
CY149465|A/greylag goose/Iceland/1482/2011|H6N8
CY167221|A/mallard/Mississippi/12OS361/2012|H11N9
CY166271|A/mallard/Wisconsin/11OS4115/2011|H11N9
CY186801|A/blue-winged teal/Ohio/12OS2321/2012|H4N6
CY134244|A/mallard/California/2589P/2011|H4N1
CY140488|A/mallard/Minnesota/Sg-00949/2008|H3N2
CY179216|A/widgeon/Wisconsin/308/1977|H6N8
CY003934|A/mallard/Alberta/323/1988|H2N1
CY005425|A/sanderling/Delaware/1258/1986|H6N6
FJ517264|A/shorebird/DE/1346/2001|H5N7
KJ195675|A/blue-winged teal/Guatemala/CIP049H105-15/2011|H14N3
KP739420|A/American green-winged teal/Washington/195750/2014|H5N1
A/Black-necked stilt/Chile/1/2013|H11N9
A/Black-necked stilt/Chile/2/2013|H11N9
GU052207|A/duck/England/1/1956|H11N6
CY130075|A/duck/Memphis/546/1974|H11N9
KJ195667|A/blue-winged teal/Guatemala/CIP049H106-62/2011|H14N6
KR824764|A/gull/Peru/PuV172/2009|H1N1
KR824691|A/ruddy turnstone/Peru/PuV181/2009|H3N1
GU050929|A/red knot/Delaware/997/2000|H10N7
CY095185|A/duck/New York/53294-6/2005|H2N3
CY067669|A/blue-winged teal/Guatemala/CIP049-01/2008|H7N9
KR824645|A/duck/Peru-MM23/2007|H4N5
CY191296|A/mallard/Maryland/13OS3035/2014|H10N7
CY096655|A/blue-winged teal/Guatemala/CIP049-05/2010|H3N8
A/Helmeted guineafowl/Colombia/1/2015|H11N2
512971|A/ruddy turnstone/DE/544/2014|H11N2
CY076442|A/American green-winged teal/California/44287-373/2007|H8N4
KR824727|A/ruddy turnstone/Peru/PuV52/2008|H12N5
CY096671|A/blue-winged teal/Guatemala/CIP049-12/2010|H5N4
CY096623|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
DQ525416|A/cinnamon teal/Bolivia/4537/2001|H7N3
CY067701|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
CY111589|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
CY067709|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
CY067693|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
CY096052|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY096060|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
0.02

Supplementary Figure S3 Phylogenic trees of PB1 (**A**), PB2 (**B**). Strains isolated in this study are indicated in black italics. Other strains characterized in this study are shown in black. AIV lineages are shown in color. Yellow, North American; Blue, South American; Green, Eurasian.


A PB1
KT002519|A/chicken/Iowa/14399-4/2015|H5N2
KT280930|A/chicken/Iowa/21981/2015|H5N2
KR492972|A/chicken/Iowa/04-20/2015|H5N2
KR233980|A/turkey/Minnesota/9892-2/2015|H5N2
KP739387|A/domestic duck/Washington/61-16/2014|H5N2
KR234028|A/chicken/Kansas/8395-3/2015|H5N2
KR150907|A/pheasant/Washington/3147-2/2015|H5N2
KR234020|A/turkey/Arkansas/7791-1/2015|H5N2
KP307974|A/Northern pintail/Washington/40964/2014|H5N2
KJ195666|A/blue-winged teal/Guatemala/CIP049H106-62/2011|H14N6
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
KR824661|A/duck/Peru/P114/2009|H3N2
KR824610|A/black skimmer/Peru/CH55/2010|H13N2
CY047566|A/mallard/Quebec/16566/2005|H11N2
CY133107|A/blue-winged teal/Wisconsin/10OS2862/2010|H3N2
CY067676|A/blue-winged teal/Guatemala/CIP049-02/2008|H7N9
KR824744|A/duck/Peru/PV80/2008|H2N9
512971|A/Ruddy turnstone/DE/544/2014|H11N2
CY167222|A/mallard/Mississippi/12OS361/2012|H11N9
CY133675|A/American green-winged teal/Mississippi/11OS250/2011|H7N3
CY143851|A/northern pintail/Interior Alaska/10BM10556R0/2010|mixed
A/Black-necked stilt/Chile/1/2013|H11N9
A/Black-necked stilt/Chile/2/2013|H11N9
CY166272|A/mallard/Wisconsin/11OS4115/2011|H11N9
KR824728|A/ruddy turnstone/Peru/PuV52/2008|H12N5
CY004317|A/mallard/Alberta/254/2003|H4N6
CY096622|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
KR824646|A/duck/Peru-MM23/2007|H4N5
CY096694|A/blue-winged teal/Guatemala/CIP049-07/2010|H8N4
CY111588|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
DQ525417|A/cinnamon teal/Bolivia/4537/2001|H7N3
CY096051|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY067692|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
CY067708|A/rosy-billed pochard/Argentina/CIP051-925/2008|H6N2
CY096059|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
CY067700|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
GU052208|A/duck/England/1/1956|H11N6
JQ906566|A/duck/Zhejiang/10/2011|H7N3
KP307982|A/gyrfalcon/Washington/41088-6/2014|H5N8
KR150899|A/turkey/California/K1500169-1.2/2015|H5N8
KF695199|A/mallard/Sweden/43/2002|H10N5
GU052516|A/turkey/England/50-92/1991|H5N1
CY005401|A/mallard/Astrakhan/266/1982|H14N5
GU052928|A/Pekin robin/California/30412/1994|H7N1
GQ414818|A/duck/Korea/112-25/2008|H6N1
HM145409|A/mallard/Jiangxi/10668/2005|H6N1
CY130076|A/duck/Memphis/546/1974|H11N9
0.02
B PB2
A/Black-necked stilt/Chile/1/2013|H11N9
A/Black-necked stilt/Chile/2/2013|H11N9
A/Helmeted guineafowl/Colombia/1/2015|H11N2
A/Helmeted guineafowl/Colombia/2/2015|H11N2
KR824701|A/ruddy turnstone/Peru/PuV182/2009|H3N1
CY096701|A/blue-winged teal/Guatemala/CIP049-08/2010|H5N3
CY125380|A/black scoter/New Brunswick/00014/2009|H7N6
CY096661|A/blue-winged teal/Guatemala/CIP049-06/2010|H8N4
FJ686801|A/mallard/Maryland/296/2003|H2N3
KR824557|A/oystercatcher/Peru/34/2006|H10N9
KR824756|A/ruddy turnstone/Peru/XXII/2006|H10N9
CY117524|A/shorebird/Delaware Bay/122/1997|H2N1
CY102607|A/pintail/Alberta/37/1999|H3N8
CY024849|A/chukar/New York/21211-7/2005|H7N2
GU051127|A/ruddy turnstone/New Jersey/748/2000|H12N5
CY096621|A/blue-winged teal/Guatemala/CIP049-10/2009|H11N2
KR824587|A/duck/Peru/CH36/2010|H11N9
KR824654|A/pelican/Peru-MM24/2007|H4N5
KR824761|A/gull/Peru/PuV172/2009|H1N1
CY067667|A/blue-winged teal/Guatemala/CIP049-01/2008|H7N9
CY096645|A/blue-winged teal/Guatemala/CIP049-04/2010|H8N4
A/ruddy turnstone/DE/544/2012|H11N2
CY167223 A/mallard/Mississippi/12OS361/2012|H11N9
CY166273 A/mallard/Wisconsin/11OS4115/2011|H11N9
CY125452|A/American wigeon/New Brunswick/04487/2007|H3N8
CY021332|A/green-winged teal/Ohio/1324/2005|H4N6
FJ686737|A/mallard/Ohio/365/2003|H2N1
GU050833|A/ruddy turnstone/New Jersey/1315/2002|H2N9
EU743120|A/pheasant/MD/4457/1993|H5N2
CY020980|A/mallard/Ohio/115/1989|H6N8
CY136661|A/mallard/Alberta/186/1987|H6N2
CY101666|A/ruddy turnstone/Delaware Bay/231/1991|H6N8
CY130077 A/duck/Memphis/546/1974|H11N9
GQ369462|A/red-winged tinamou/Argentina/MP1/2008|H1N1
CY067691|A/rosy-billed pochard/Argentina/CIP051-557/2007|H6N2
CY096050|A/rosy-billed pochard/Argentina/CIP051-1977/2010|H6N2
CY096058|A/rosy-billed pochard/Argentina/CIP051-269/2007|H6N8
CY067699|A/rosy-billed pochard/Argentina/CIP051-575/2007|H6N8
DQ525418|A/cinnamon teal/Bolivia/4537/2001|H7N3
CY111587|A/rosy-billed pochard/Argentina/CIP051-559/2007|H9N2
GU052209 A/duck/England/1/1956|H11N6
CY030244|A/duck/Vietnam/NCVD-2/2007|H5N1
EF178510|A/tree sparrow/Thailand/VSMU-16-RBR/2005|H5N1
KP097839|A/duck/Vietnam/NCVD-KA368/2012|H5N1
GU186691|A/Duck/Hong Kong/380.5/2001|H5N1
KP307981|A/gyrfalcon/Washington/41088-6/2014|H5N8
KR234011|A/turkey/Missouri/7458-1/2015|H5N2
KT002486|A/chicken/Iowa/13542-2/2015|H5N2
KR234003|A/turkey/Minnesota/7172-1/2015|H5N2
KR234027|A/chicken/Kansas/8395-3/2015|H5N2
KR234019|A/turkey/Arkansas/7791-1/2015|H5N2
KT002470|A/turkey/Iowa/11762-1/2015|H5N2
KR234035|A/turkey/Minnesota/9845-4/2015|H5N2
0.02

CHAPTER 4

PREVALENCE, DIVERSITY AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES OBTAINED FROM WILD BIRD SURVEILLANCE IN CHILE

Pedro Jiménez-Bluhm, Erik A. Karlsson, Pamela Freiden, Bridgett Sharp, Jorge E. Osorio, Christopher Hamilton-West and Stacey Schultz-Cherry

ABSTRACT

While the circulation of avian influenza viruses (AIVs) has been well documented in most continents, data from South America is still sparse. To address this gap in knowledge, AIV surveillance efforts were carried out between 2012 and 2015 in Chile. A total of 3718 environmental fecal samples were collected from wild birds and screened for AIV, resulting in an overall AIV prevalence of 2.9%. Subtypes H1N1, H3N6, H4N2, H4N6, H5N3, H7N3, H7N6, H9N2, H9N7 and H11N9 were isolated from 12 different bird species, making this the largest and most diverse collection of AIV obtained in Chile to date. Phylogenetic analysis shows co-circulation of both North American and novel South American AIV lineages as well as the formation of interhemispheric reassortants. In a mouse challenge, neither of the novel H5, H7 nor H9 viruses produced significant weight loss, indicating the low risk these Chilean strains pose to mammals. The discovery of a broad array of AIV subtypes, including some of public health concern, highlights the importance of avian influenza surveillance in wild birds in Chile.

BACKGROUND

Wild birds play a central role in the transmission of AIV and several long term surveillance studies have provided valuable information about AIV distribution and evolution, leading to a great understanding about these dynamics in the northern hemisphere (1–3). However, there is a dearth of knowledge regarding the prevalence and diversity of AIV in South America, mainly due to a lack of sustained surveillance efforts in the region (4). This is of particular concern, since intercontinental migration of birds can facilitate the dispersion of pathogens, particularly those that do not significantly affect their host's health status, like avian influenza (1). Previous reports of AIV in Chile are limited to an HPAI outbreak of H7N3 virus in commercial poultry and to the isolations of three low pathogenic avian influenza (LPAI) subtypes of North American origin in shorebirds (5,6). This has led to a general believe that the presence of AIV in wild birds in Chile is negligible and sporadic (7,8).

To date, the exchange rate of AIV between North and South America is unknown. Surveillance studies performed in Argentina have shown the widespread presence of a South American sublineage of AIV, with little interhemispheric exchange of gene segments (9,10). On the contrary, analysis of Colombian, Brazilian and Peruvian isolates have revealed genome constellation composed almost exclusively of North American-type AIVs (11–13). Even though these surveillance efforts have provided a partial picture of the ecology and distribution of the AIV in the South America, most of them lack formal risk assessment studies. Other than limited studies of virulence in chickens performed on Chilean H7N3 and Argentinian H9N2 viruses, little is known about the potential risk that these emerging South American viruses represent to both the human and animal populations (14,15). This is of particular concern, since H5, H7 and H9 AIV

subtypes of avian origin have been implicated in several cases of infection and disease in humans and animals (16–18). Despite these pathogenic subtypes circulating in Asia and Europe and the constant threat of introduction of these viruses by wild birds into the American continent (19,20), knowledge about the genetic and phenotypic relationships of those pathogenic viruses to circulating strains in South America is insufficient.

Given the worldwide distribution of AIV and its transmission trough wild birds (3), we challenge the hypothesis that AIV presence in Chile is insignificant. In order to gain further insight into the presence of this pathogen in the country, an active AIV surveillance study was carried out between 2012 and 2015. Efforts were focused on understanding the spatial and temporal distribution of AIV, as well as to comprehend the origin, diversity and pathogenic potential of this infectious agent in wild birds in Chile. Of 3718 collected samples, we detected 108 AIV positive samples of which we obtained 16 viral isolates. This represents the largest and most diverse collection of AIVs acquired in Chile to date. Sequence and phylogenetic analysis revealed several interhemispheric reassortment events. Most subtypes displayed several decades of divergence from contemporary North American wild bird strains, providing additional evidence of a South American lineage of AIV. Finally, mice infected with native H5, H7 and H9 subtypes developed little to moderate morbidity, suggesting a limited pathologic potential of these Chilean wild bird viruses in mammals.

METHODS

Ethics Statement

All sampling activities and animal experiments were approved by the St Jude Children's Research Hospital Institutional Animal Care and Use Committee (IACUC). For sampling at La Farfana lagoon, permission was obtained from Aguas Andinas S.A.

Sample sites and sample collection

Between June 2012 and September 2015, 16 sample sites (Figure 1) were visited and 3718 fresh wild bird feces were collected. Sites consisted of wetlands, shorelines, estuaries and lagoons and were selected based upon access and significant bird presence. Sample collection was carried out during 5 sampling seasons: June to July 2012 (n=262), March 2013 (n=357), November 2013 (n=944), April 2014 (n=1138) and September 2015 (n=1017). Collection was done using single-use sterile swabs and placed in cryovials containing 1 ml phosphate-buffered saline with 50% glycerol and antibiotics (penicillin 10000 IU/ml, streptomycin 5 mg/ml, gentamicin sulfate 1 mg/ml, neomycin sulfate 700 µg/ml and amphotericin B 10 µg/ml; Sigma Chemical Co, St. Louis, MO, USA) or using 1 ml Universal Transport Media, UTM^{TM} (Copan Italia S.P.A). Samples were kept at 4^{o}C for a maximum of 4 days, then stored at -80^{o}C until analysis.

Sample screening and virus isolation

Following previous described methodology (13), viral RNA was extracted from 50 ul fecal sample on a Kingfisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, USA) by using the Ambion MagMAX-96 AI/ND Viral RNA Isolation kit (Life Technologies Corporation, Grand

Island, NY, USA). RNA was screened using a Bio-Rad CFX96 Real-Time PCR Detection System on a C1000 Thermocycler (Bio-Rad, Hercules, CA, USA), with TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA) and primers/probe specific for the influenza M gene (21). Samples with a fluorescence cycle threshold value (Ct.) <38 were considered positive. Virus isolation was attempted on all samples with a Ct. <35 in embryonated chicken eggs as previously described (22). Isolates were confirmed by hemagglutination assay (HA) and rRT-PCR, and viral titers determined by Reed and Munch by 50% tissue culture infectious dose ($TCID_{50}$) in Madin-Darby canine kidney cells (MDCK) and 50% egg infectious dose (EID_{50}) (23). Viruses were stored at -80°C.

Virus sequencing

Reverse transcription of viral RNA was performed using SuperScript Vilo™ (Life Technologies Corporation, Grand Island, NY, USA). Amplicons were obtained using Phusion High-Fidelity DNA polymerase and Q5 High-Fidelity DNA Polymerase (New Ingland BioLabs, Ipswich, MA, USA) with universal oligonucleotide primers as described (24,25). Amplicons were later purified by agarose gel electrophoresis, purified using Zymoclean™ Gel DNA Recovery (Zymo Research Corporation Irvine, CA, USA) and full-length gene segments ligated into the pCR™-Blunt II-TOPO® (Life Technologies Corporation, Grand Island, NY, USA) and amplified in HB101 *E. coli* strain (Zymo Research Corporation, Irvine, CA, USA). Minipreps were performed using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Smaller gene fragments produced by the HA1134F/HA-NS 890R primer combination were sequenced directly after gel purification (25). Sequencing was performed by Sanger sequencing at the University of Wisconsin-Madison Biotechnology Center. Gene segment similarities were analyzed by BLAST (26). Host species were identified using primers designed to amplify a segment of the mitochondrial cytochrome-

oxidase I (COI), as described (27). Obtained sequences can be accessed under Genbank numbers KX1011143 to KX101207 and KX185892 to KX185931.

Phylogenetic analysis

Sequence assembly and editing was performed using BioEdit version 7.2.5 (28). Sequence alignment was executed using MUSCLE version 3.8.3 (29). Genome searches and obtainment of reference sequences for the alignments was done through the Influenza Virus Resource at NCBI (30). Phylogenic relationships for each gene was inferred by Maximum Likelihood, incorporating a general time-reversible model of nucleotide substitution with a gamma-distributed rate variation among sites using RaxML version 8.0 (31). Time-scaled phylogenic analysis was performed with the BEAST package version 1.8.2 using the HKY85 substitution model, time-stamped sequence data and a lognormal relaxed-clock Bayesian Markov chain Monte Carlo method (32–34). Hemagglutinin (HA) subtypes H1, H3, H4, H5, H7, H9 and H11 and neuraminidase (NA) N6 were analyzed by this methodology. The BEAGLE library version 1.6 was used for optimization of computational power (35) . The starting tree was selected randomly for each run and we performed at least three independent analysis of 50 million generation each using a Bayesian skyline coalescent tree prior model and 10 coalescent groups. Runs were combined after removing 10% of the burn-ins for each analysis and combined using LogCombiner version 1.8.2. Coalescence of the runs was visually monitored using Tracer version 1.6 with an expected effective sample size for each statistic of at least 200. FigTree version 1.4.2 was used for visualization of the annotated phylogenetic trees. Node ages were estimated by analyzing the times-scaled maximum clade credibility (MCC) trees.

Animal infections

6- to 8-week-old female BALB/c mice (n=11) (Jackson Laboratory, Bar Harbor, ME) were lightly anesthetized with isoflurane and intranasally inoculated with 10^4 $TCID_{50}$ of virus in 25 µl PBS. Mice were monitored daily for clinical signs of morbidity, like lethargy and body weight loss (36). Mice were weighed every 24 h (36). At day 3 and 6 pi, n=3 mice were euthanized and nasal washes and lungs were harvested for viral titers by $TCID_{50}$.

Statistical Analysis

Data management, viral prevalence, descriptive statistics and confidence intervals were calculated using an Excel 2013 worksheets (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Prevalence, viral diversity and host species

In order to evaluate viral prevalence in Chile, we collected 3718 fresh bird fecal samples from June 2012 to September 2015, during 5 sampling seasons at 16 sites (Figure 1). Samples sites ranged from northern to central Chile, and included wetland, estuaries, shorelines and lagoons. In total, 108 positive rRT-PCR samples, 16 AIV isolates and 12 partial genomes were obtained. Overall, AIV prevalence throughout all sampling seasons was of 2.9% (95% CI, 2.36% to 3.44%), ranging from 0.42% (95% CI, 0% to 1.13%) in 2012, to 2.3% in 2013 (95% CI, 1.38% to 3.24%), 4.4% in 2014 (95% CI, 3.2% to 5.58%) and 2.65% (95% CI, 1.67% to 3.64%) in 2015. Of the 16 sample sites, only 9 (56.3%) yielded positive AIV samples throughout the study. The most regularly visited sites were Batuco (n=879), La Farfana (n=153), Mantagua (n=827) and Llolleo

(n=586). The highest AIV prevalence at Batuco was detected in October (spring) 2015 with 4.6% (95% CI, 1.28% to 8%), in La Farfana in April (fall) 2014 with a 3.9% (95% CI, 0% to 11.24%), in Mantagua in April 2014 with a 4.2% (95% CI, 2.28% to 6.12%) and in Llolleo in March (summer) 2013 with a 7.6% (95% CI, 2.53% to 12.67%). Interestingly, the northern Lluta wetland, located next to the Chilean-Peruvian border, displayed a prevalence of 5.2% (95% CI, 2.31% to 8%) during spring 2015 (Figure 2). However, this was the only time this site was visited; hence no trend in AIV prevalence could be established. The lowest AIV prevalence was obtained during the sampling season of winter 2012 (June and July), with La Farfana being the only site yielding positive wild bird samples during this period, reaching a prevalence of 2.7% (95% CI, 0% to 8.15%) (Figure 2). The low AIV prevalence in winter and its subsequent rise during spring could indicate that AIV keeps circulating in wetlands and resident wild birds in Chile before migratory birds arrive from northern hemisphere. The highest display of AIV prevalence at the end of migratory season is also in agreement with literature that typically identifies late summer and early fall as the period with the highest AIV prevalence (3).

Diverse HA and NA subtypes were obtained including 10 different HAs and 6 NAs, yielding 11 distinct subtype combinations (Table 1). Most of these AIV subtypes were obtained from *Anseriformes;* particularly, Yellow-billed pintails (*Anas georgica*) and Yellow-billed teals (*Anas flavrostris*), which were responsible for carrying most of the subtype diversity (Table 2). Both duck species are typical throughout South America and particularly abundant at Chilean wetlands (37). Other waterfowl species identified as AIV reservoirs were mallards (*Anas platyrhynchos*), Chiloé Wigeons (*Anas sibilatrix*) and Red-fronted coots (*Fulica rufifrons*). Birds belonging to order *Charadriiformes* were also identified as AIV reservoirs. Of these, we identified Black necked stilts (*Himantopus melanurus*), Whimbrels (*Numenius phaeopus*), Kelp gulls (*Larus*

dominicanus), Franklin's gulls (*Leucophaeus pipixcan*), Gray plovers (*Pluvialis squatarola*), American oystercatchers (*Haematopus palliatus*) and Blackish oystercatchers (*Haematopus ater*) (Table 2).

Phylogenic and genetic characterization of Chilean sequences

Divergence time between the North and South American clade is unknown for most HA subtypes of South American origin, therefore we performed a molecular clock analysis of the isolated HAs and compared their nucleotide (NT) and amino acid (AA) similarity to publicly available sequences. Bayesian molecular clock analysis of subtypes of public health concern circulating in Chile, e.g., H5, H7 and H9, reveals that they form distinct and recognizable lineages, different to contemporary wild bird North American and Eurasian viruses.

H5 Analysis if the novel H5 subtype (A/Yellow-billed pintail/Chile/C1267/2015, hereafter named YBP/H5N3) shows that the divergence time from the North American clade is situated in 1982 (95% Bayesian confidence interval (BCI) 1978-1986). By comparing the H5 subtype to publicly available sequences, the highest NT and AA sequence similarity between YBP/H5N3 and its closest relative, A/mallard/Ohio/556/1987(H5N9), is 90% NT and 95% AA (Figure 3, Table 3).

H7 Phylogeny shows that the H7 subtype has a long divergence time from the North American lineage, with the time of most common ancestor (TMRCA) situated in 1958 (95% BCI 1948-1968). The hemagglutinins of the H7N3 and H7N6 viruses (A/Yellow-billed pintail/Chile/10/2014 and A/Yellow-billed teal/Chile/8/2013, hereafter named YBP/H7N3 and YBT/H7N6), were closest related to a South American AIV, A/cinnamon teal/Bolivia/4537/2001(H7N3), and shared a 93% NT and 96% AA similarity to it (Figure 3, Table 3).

H9 The H9 subtype's TRMCA is situated in 1985 (95% BCI 1978-1991). Both HAs of the H9N2 and H9N7 subtype (A/American oystercatcher/Chile/C1307/2015 and A/Grey

Plover/Chile/C1313/2015, hereafter named AOY/H9N2 and GPL/H9N7) are closely related to a South American isolate, A/rosy-billed pochard/Argentina/CIP051-559/2007(H9N2), with a 94% NT and 98% AA similarity (Figure 3, Table 3).

With the exception of the H4 and H11 subtypes, all other HAs analyzed in this study display some degree of South American divergence, and the TMRCA of these subtypes varies when compared to publicly available North American sequences (Figure 4, Table 3).

H1 The HA of the H1N1 isolate is closest related to an Argentinean isolate obtained previously from a flightless tinamou (*Rhynchotus rufescens*) (38). These two HAs share a 98% nucleotide identity and diverged from the North American lineage as recently as 1979 (95% BCI 1970 - 1988) (Figure 4, Table 3). By contrast, the closest North American sequence, A/pintail duck/ALB/238/1979 (H1N1), shows only 90% nucleotide and 96% amino acid similarity.

H3 This subtype was the most divergent sequence obtained in this study and did not belong to an avian lineage of AIV, but was related to an H3N8 equine influenza virus obtained in Miami in 1963 (A/equine/Miami/1/1963) (Figure 4, table 3). It shares an 86% to 92% and NT and AA similarity to this sequence and diverged from the North American clade in 1914 [95% BCI 1874 – 1945].

Of the neuraminidases, the N6 subtype displayed unique characteristics never described before, as it formed a separate lineage when compared to North American and Eurasian segments. In order to assess the age of this novel sublineage, we performed a molecular clock analysis of this segment. Bayesian analysis reveals that the N6 TMRCA is situated in 1927 (95% BCI 1907-1950) (Supplementary Figure S1). The closest available to the N6 sequence, A/pintail duck/ALB/159/1977 (H4N6), had an 81% NT and 90% AA identity.

In order to establish the occurrence of interhemispheric reassortment events, we compared all obtained segments to AIV sequences of North American and Eurasian origin allowing us to establish genome similarities by phylogenic analysis (Table 4). According to our results, the more diverse viruses obtained in this study were AOY/H9N2 and GPL/H9N7, due to their high reassortment rate between lineages (Table 4).

In terms of molecular markers of pathogenicity, the deduced amino acid sequence of the HA cleavage site of all isolated viruses was consistent with LPAI viruses, limiting their replication to trypsin rich tissue, like the gastrointestinal track of birds (Table 5) (39). We were unable to detect any markers associated with increased transmission to mammals, e.g., PB1-627K or PB2-701N; nor antiviral resistance (40,41).

Chilean isolates produced moderate morbidity in mice

Little is known about the pathogenic potential of South American AIV isolates in mammals, and no studies have tested the pathogenicity of viral strains isolated in Chile in a mouse model before. In order to assess the pathogenicity of YBP/H5N3, YBP/H7N3, YBT/H7N6, AOY/H9N2 and GPL/H9N7 in mice, we intranasally infected 6 to 8 week old Balb/c mice with $10^4 TCID_{50}$ of each virus. We monitored weight loss and sign of morbidity until day 14 post infection (pi). In average, none of the viruses produced weight loss greater than 10% of their original weight and mice began to gain weigh consistently after day 6 pi (Figure 5A). However, one mouse infected with H7N6 lost up to 14% of its weight on day 4 pi. Similarly, one mouse of the H7N3 infected group loss up to 17% of its weight on day 4pi. All mice but one of the GPL/H9N7 group survived until day 14 post infection (Figure 5B). This mouse begun losing weight on day 3 pi and had to be euthanized due to a 30% weight loss by day 5 pi.

DISCUSSION

Avian influenza prevalence is known to vary according to seasons. This has been demonstrated by studies performed in North America, were the estimated AIV prevalence in ducks varies between less than 1% in spring to up to 60% during late summer and fall, with similar results also reported in Europe (1,2,42). However, there is limited information about the prevalence of AIV in wild birds in South America (4). The peak of prevalence in the northern hemisphere during fall is believed to be related to a high number of immunologically naïve birds born that season and to the high contact rate between birds during marshalling (1–3). Results in Chile are consistent with these reports. In general, highest AIV prevalence was reported during summer and fall in La Farfana, Llolleo and Mantagua. On the contrary, Batuco showed highest prevalence during spring 2013 and 2015. This difference could be attributed to a lack of continuous surveillance, making it hard to reach any conclusions as to whether there are any particular circumstances surrounding a higher spring prevalence at this site. Sample sites on the coast between Managua and Llolleo were heavily degraded due to urbanization and habitat contamination. These sites were visited during the exploratory phase of winter 2012 and yielded no positive samples. Due to an inconsistent and low presence of birds, these sites were not visited on consecutive sampling seasons.

Subtype diversity was shared between *Anseriformes* and *Charadriiformes*, being the H9 and H13 subtypes restricted to the latter. Our findings differ partly from literature, since the H9 subtype has also been recovered from wild ducks around the world in some occasions (15,43). However, the reason behind this discrepancy is most likely due to the reduced sample size and paucity of the field seasons of our study and not to an inherent difference in bird susceptibility to AIV in Chile. Only a single isolate was obtained from coots (*Rallidae*). This is not infrequent, since coots are not considered a common reservoir of AIV (2).

The results of this research are unique, since they show for the first time that a great diversity of AIVs are circulating in waterfowl and shorebirds in Chile. Results also demonstrate that interhemispheric exchanges of AIV in the region are frequent. This suggests that Chile is an intermediate area, where North and South American AIVs frequently intermix, contrary to what has been reported in neighboring countries, like Argentina and Peru (9–11,15,38). Given our findings, it appears that bird migration is partly responsible for the independent evolution of AIV in South America. Most of the isolated strains were obtained from native duck species with a host range limited to the Southern Cone (Chile, Argentina, Uruguay, and Southern Brazil) (44). This information is important for various reasons. So far, the reservoir species of AIV in Chile were unknown, hence risk based surveillance based on species was impossible. For example, in Central America and northern South America, the presence of Blue-winged Teals (*Anas discors*) is used as an indicator of possible AIV presence (45,46). Host information also provides an important tool in order to focus sampling efforts, by narrowing down target species (46). Regrettably, even though the observed host range is relatively established for the species described as AIV carriers in this study (44), movement of these species within their range is not well established, making it hard to evaluate viral flow within the region. Nevertheless, the endemism of these waterfowl species within a geographically segregated area like the Southern Cone, most probably have contributed to the allopatric speciation of AIV in the region. Of the identified host species only the Whimbrel, Gray Plover and Franklin's gull have an interhemispheric migratory behavior (44) but in this study the subtypes obtained from these species are mostly associated to *Charadriiformes*. Thus shorebirds and gulls apparently do not play a major role in the overall diversity of subtypes seen in wild birds in Chile.

While AIV gene pool segregation due to geographical barriers is easy to understand between North America and Europe or Asia, it is harder to explain between AIVs of North and South American origin. Reassortment is known to occur at low frequencies between the North American and the Eurasian AIV lineages (47–49) and more surveillance data is needed in South America in order to estimate such events. Reassortment of internal genes and surface proteins from North and South American lineages has been documented a few times before in South America (10,15,38), but viruses isolated from wild birds in Chile to date have only yielded North American-like viruses (5). Results show that the obtained sequences belong to either North or South American lineages. In general, most AIVs show interhemispheric reassorment events, with the H3N6 and H11N9 viruses the only exceptions. Also, Bayesian analysis revealed a long divergence time of most surface proteins from their North American ancestors. This had been previously reported for the H7 subtype of South American origin, but not for the H5 subtype (50). The age of introduction of the H7 subtype established by us is similar to previous estimates that have situated the South American H7 TMRCA in 1955 (95% BCI 1938-1969) (50). This study also describes the first South American-like N6, as a previous N6 obtained in Peru belongs to the North American AIV lineage (11).

The introduction and co-circulation of distinct viral genomes gives the opportunity for the formation of reassortants and the generation of novel AIVs. Based on our results, the far north of Chile is more exposed to North American AIVs compared to central Chile. This can be assessed by the higher reassortment rate of AOY/H9N2 and GPL/H9N7 compared to isolates from the central region of Chile. By analogy, the farther south of Chile should see less influx of North American gene segments and a greater presence of the South American clade. In the wake of the recent introduction of the HPAI H5 clade 2.3.4.4 of Asian origin into wild birds and poultry in

North America (51), this information can be important in order to establish early warning systems based on AIV surveillance in the northern part of the country, like the Lluta wetland (9,11). Altogether, the endemism and novelty of certain gene segments, as well as the frequent reassortment events between lineages, are an indicator of the particular characteristics of AIV ecology in Chile. For example, the H1 subtype had a serine to alanine substitution in the second position of the HA cleavage site PSIQ**A**R/GLF. This substitution appears to be exclusive of South American H1 viruses (38), but the significance of this mutation is unknown. This HA sublineage is uncommon and is shared by only one more sequence obtained previously from a flightless bird in Argentina (38). Moreover, in addition to avian-origin influenza A, we also detected a H3 hemagglutinin related to the so-called equine-2 influenza viruses (3). There has been no report of an avian "equine-like" HA in South America before, and this sequence could be related to the origins of the equine H3N8 pandemic of the 60's, believed to have originated in South America (52).

The emergence of AIV in poultry and transmission to mammals, including swine and humans, is unpredictable. Of great concern are H5, H7 and H9 AIVs, that are known to transmit from birds to humans (16,18). In particular, the H7N3 subtype has repeatedly become HPAI in poultry in the Americas and caused disease in humans (53,54). However, although individual mice infected by the H7 subtype developed some level of morbidity, we did not find any of these three subtypes to be highly pathogenic in mice. Unsurprisingly, this indicates that AIV viruses obtained from wild birds in Chile are still mostly avian adapted and may pose no immediate threat to mammals. The moderate morbidity seen in mice could also be due to the relatively low viral titers of the isolates, as virus isolates were minimally passaged in order to avoid mutations associated with egg adaptations. Finally, other AIV isolates with potential to infect mammals, like H1N1,

H4N2/H4N6 and H11N9 were also obtained in this study. H1N1 AIV obtained from wild birds has shown to be able to transmit between ferrets (55) and evidence of seroconversion against H4 and H11 subtypes has been reported in Lebanese poultry growers (56). Many of the isolates obtained in this study should therefore explored further to better understand their potential to infect mammals.

In summary, we describe the presence of a wide array of AIV subtypes isolated from wild ducks and shorebirds in Chile, their evolutionary pathways, and their potential of infection in mice. Further studies are needed to better elucidate de ecology of AIVs in Chile, to establish prevalence, AIV host range and pandemic potential trough thoroughly planned risk based surveillance efforts.

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Author Contribution:

Jimenez-Bluhm, P (self) Designed sampling efforts and performed sample screening and viral isolation. Performed sequencing and phylogeny. Organized and conctructed figures. Wrote manuscript.

Karlsson, EA Performed mouse experiments.

Freiden, P Assisted in sample screening, provided technical assistance.

Sharp, B Assisted in sample screening, provided technical assistance.

Hamilton-West, C: Assisted in preparation of manuscript. Edited manuscript. Constructed Figure 1.

Schultz-Cherry, S Assisted in designing sampling strategies. Edited manuscript.

Osorio, JE Assisted in designing sampling strategies. Assisted greatly in the preparation of manuscript and its editing.

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Figure 1 Location of sample sites. Positive sites in red, negative sites in blue. Subtypes obtained described per site.

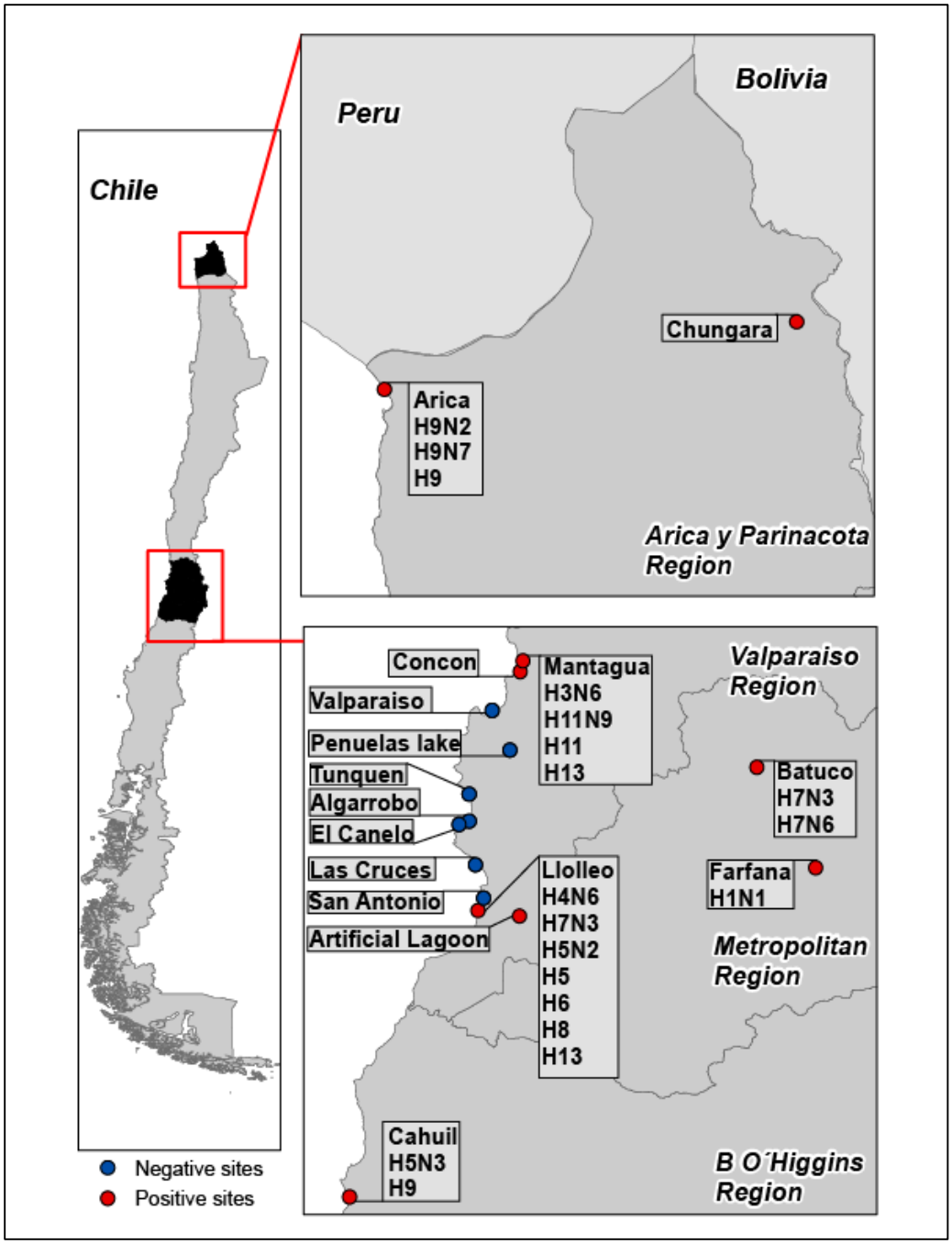

Chile
Peru
Bolivia
Chungara
Arica
H9N2
H9N7
H9
Arica y Parinacota
Region
Concon
Mantagua
H3N6
H11N9
H11
H13
Valparaiso
Region
Valparaiso
Penuelas lake
Tunquen
Algarrobo
El Canelo
Batuco
H7N3
H7N6
Las Cruces
San Antonio
Artificial Lagoon
Llolleo
H4N6
H7N3
H5N2
H5
H6
H8
H13
Farfana
H1N1
Metropolitan
Region
Cahuil
H5N3
H9
B O´Higgins
Region
Negative sites
Positive sites

Figure 2 AIV prevalence per site per season. Columns show prevalence and bars indicate upper 95% CI; (*) data not available; (**) information of only one season available.

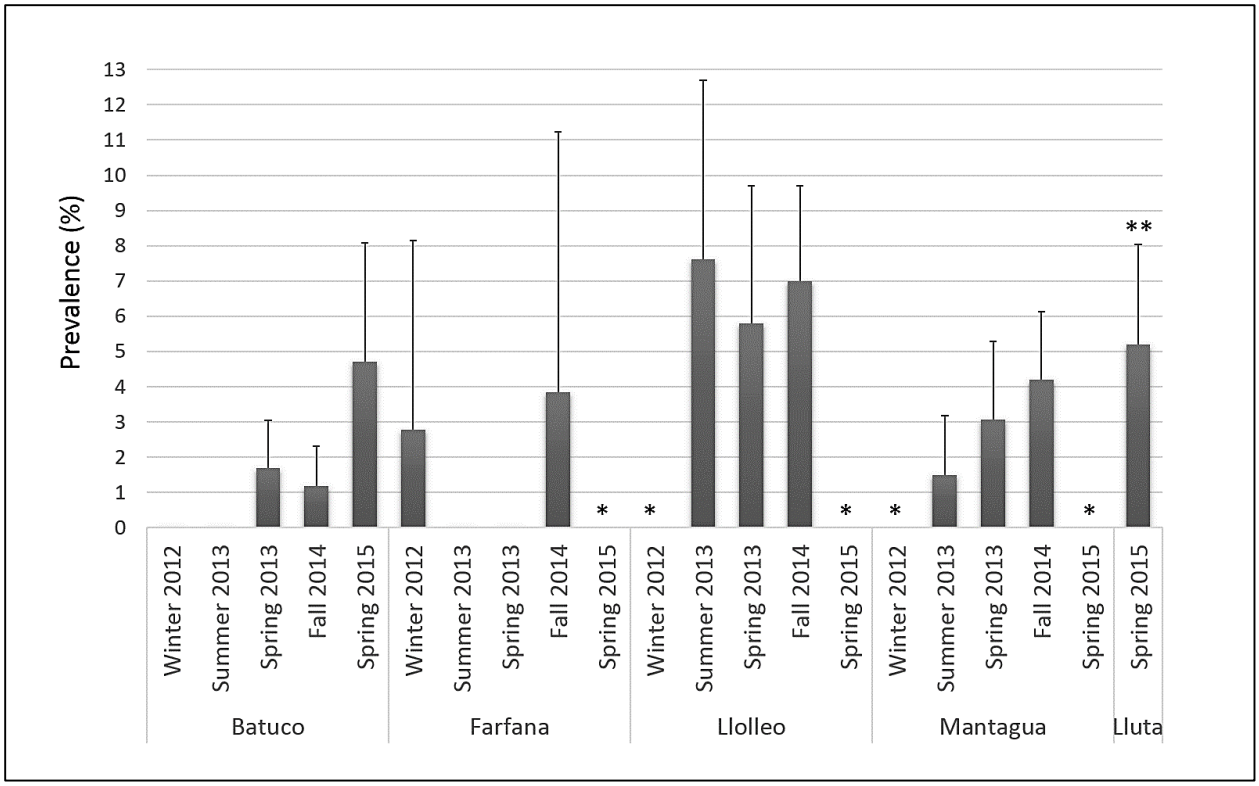

Prevalence (%)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
Winter 2012
Summer 2013
Spring 2013
Fall 2014
Spring 2015
Batuco
Farfana
Llolleo
Mantagua
Lluta
*
**

Table 1 HA and NA subtypes reported by this study.

	HA																
NA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	NA Total
1	1[i]																1
2				2[i]	1				1[i]								4
3					1[i]		3[i]										4
4																	
5																	
6			1[i]	2[i]			2[i]										5
7									1[i]								1
8																	
9											2[i]						2
x					3	1	1	2	3		1		2				13
Total HA	1		1	4	5	1	6	2	5		3		2				30

X[i] indicates viral isolate

Table 2 Birds tested positive to avian influenza and obtained subtypes. Chile, 2012-2015.

Host species	**Order**	**AIV Subtype**
Chiloé wigeon *(Anas sibilatrix)*	*Anseriformes*	H5N2
Mallard (*Anas platyrhynchos*)	*Anseriformes*	H4N2
Yellow-billed pintail *(Anas georgica)*	*Anseriformes*	H1N1, H4N2, H4N6, H5N3, H7N3
Yellow-billed teal (*Anas flavirostris*)	*Anseriformes*	H7N3, H7N6, H5Nx, H6Nx, H8Nx, H11Nx
Red-fronted coot (*Fulica rufifrons)*	*Gruiformes*	H3N6
American oystercatcher (*Haematopus palliatus*)	*Charadriiformes*	H9N2
Blackish oystercatcher (*Haematopus ater*)	*Charadriiformes*	H9Nx
Black necked stilt *(Himantopus mexicanus)*	*Charadriiformes*	H11N9, H11Nx
Franklin's gull (*Larus pipixcan*)	*Charadriiformes*	H13Nx
Gray plover (*Pluvialis squatarola*)	*Charadriiformes*	H9N7
Kelp gull (*Larus dominicanus*)	*Charadriiformes*	H13Nx
Whimbrel (*Numenius phaeopus*)	*Charadriiformes*	H9Nx

(x) indicates unknown NA subtype.

Table 3 HA genetic diversity analysis. Closest North American sequence, NT and AA similarity (%) and within subtype TMRCAs for each HA subtype are indicated.

Subtype	**Closest North American sequence** **[NT - AA similarity %]**	**TMRCA** **(mean [95% BCI])**
H1	A/pintail duck/ALB/238/1979 (H1N1) [90 - 96]	**1979** [1970 - 1980]
H3	A/equine/Miami/1/1963(H3N8) [86 - 92]	**1914** [1874 – 1945]
H4	A/blue winged teal/Minnesota/AI09-2977/2009(H4N8) [96 - 99]	**2008** [2007 – 2009]
H5	A/mallard/Ohio/556/1987(H5N9) [90 - 95]	**1982** [1978 – 1986]
H7	A/turkey/Oregon/1971(H7N3) [83 - 90]	**1958** [1948 – 1968]
H9	A/shorebird/Delaware Bay/260/1996(H9N9) [90 - 95]	**1985** [1978 – 1991]
H11	A/ruddy turnstone/DE/544/2014 [99 - 99]	**2012** [2012 – 2013]

Table 4 Genetic diversity of Chilean avian influenza viruses obtained in Chile. Boxes indicate gene segment position as resolved by Maximum Likelihood analysis. Dark-gray, North American avian; light-gray, South American avian; black, equine. Alleles for the NS gene indicated as “A” or “B”.

Virus	Subtype	PB 2	PB 1	PA	HA	NP	NA	M	NS
A/Yellow-billed pintail/Chile/1/2012	H1N1								B
A/Red-fronted coot/Chile/5/2013	H3N6								A
A/Yellow-billed pintail/Chile/6/2014	H4N6								A
A/Yellow-billed pintail/Chile/7/2014	H4N6								A
A/Yellow-billed teal/Chile/C918/2015	H4N2								A
A/Mallard/Chile/C948/2015	H4N2								A
A/Chiloe wigeon/17/2014	H5N2								
A/Yellow-billed pintail/Chile/C1267/2015	H5N3								A
A/Yellow-billed teal/Chile/13/2014	H5Nx								
A/Yellow-billed teal/Chile/14/2014	H5Nx								
A/Yellow-billed teal/Chile/15/2014	H5Nx								
A/Yellow-billed teal/Chile/16/2014	H6Nx								
A/Yellow-billed teal/Chile/8/2013	H7N6								B
A/Yellow-billed teal/Chile/9/2013	H7N6								B
A/Yellow-billed pintail/Chile/10/2014	H7N3								A
A/Yellow-billed pintail/Chile/11/2014	H7N3								A
A/Yellow-billed teal/Chile/12/2014	H7N3								B
A/Yellow-billed teal/Chile/C847/2015	H7Nx								A
A/Yellow-billed teal/Chile/21/2014	H8Nx								
A/Yellow-billed pintail/Chile/C945/2015	H8Nx								
A/American oystercatcher/Chile/C1307/2015	H9N2								A
A/Grey plover/Chile/C1313/2015	H9N7								A
A/Whimbrel/Chile/C1274/2015	H9Nx								
A/Whimbrel/Chile/C1357/2015	H9Nx								
A/Blackish oystercatcher/Chile/C1403/2015	H9Nx								
A/Black-necked stilt/1/2013	H11N9								A
A/Black-necked stilt/2/2013	H11N9								A
A/Yellow-billed teal/Chile/4/2013	H11Nx								
A/Franklin's gull/Chile/3/2013	H13Nx								
A/Kelp gull/Chile/18/2014	H13Nx								

Figure 3 Maximum clade credibility trees of H5, H7 and H9 subtypes. Sequences obtained in this study were compared to South American, North American and Eurasian sequences of avian and swine origin. (*) indicates sequences obtained during this study.

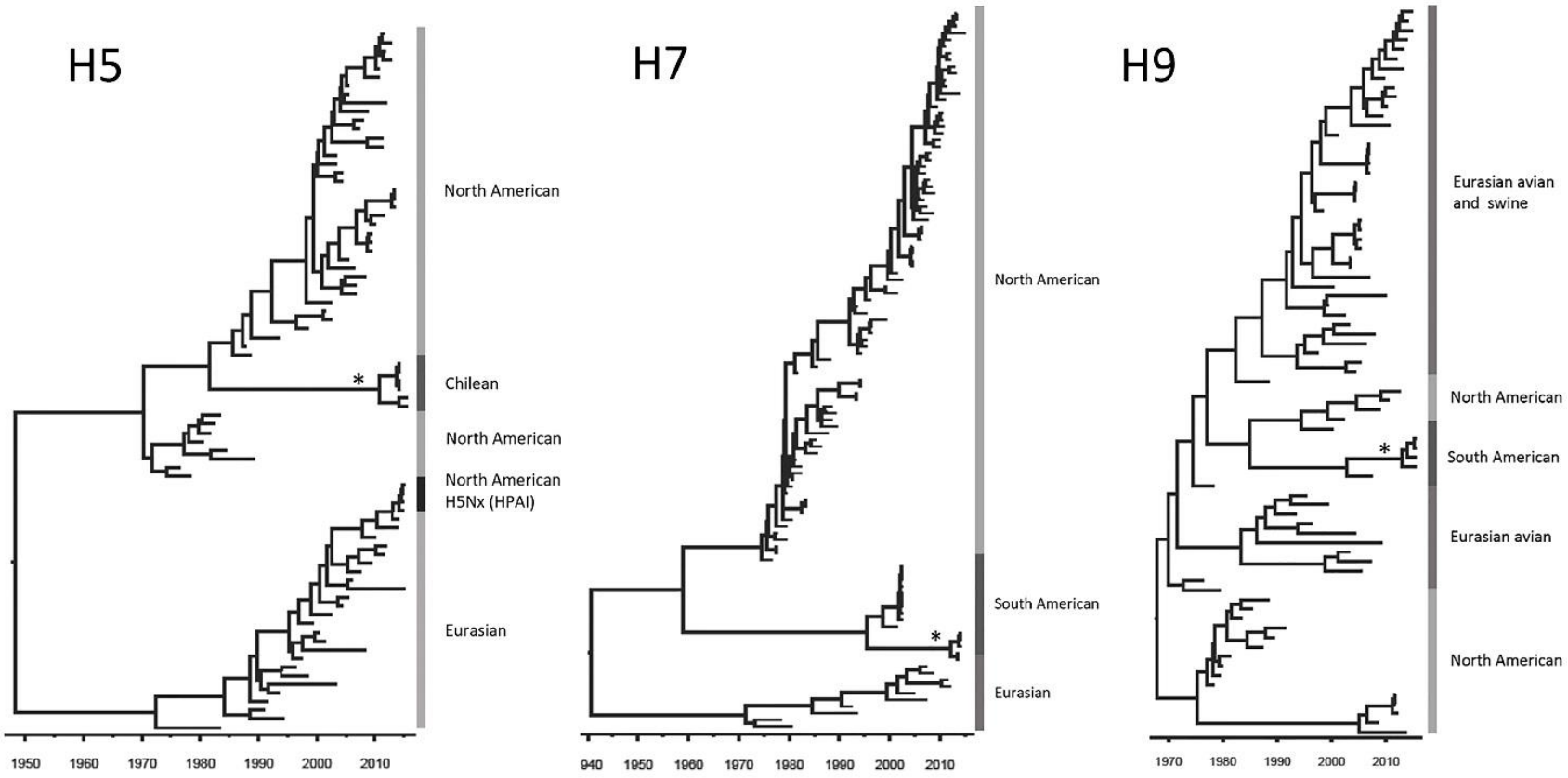

H5
North American
Chilean
North American
North American
H5Nx (HPAI)
Eurasian
1950
1960
1970
1980
1990
2000
2010
H7
North American
South American
Eurasian
940
1950
1960
1970
1980
1990
2000
2010
H9
Eurasian avian
and swine
North American
South American
Eurasian avian
North American
1970
1980
1990
2000
2010

Figure 4 Maximum clade credibility trees of H1, H3, H4 and H11 subtypes. Sequences obtained in this study were compared to South American, North American and Eurasian sequences of avian and equine origin. (*) indicates sequences obtained during this study.

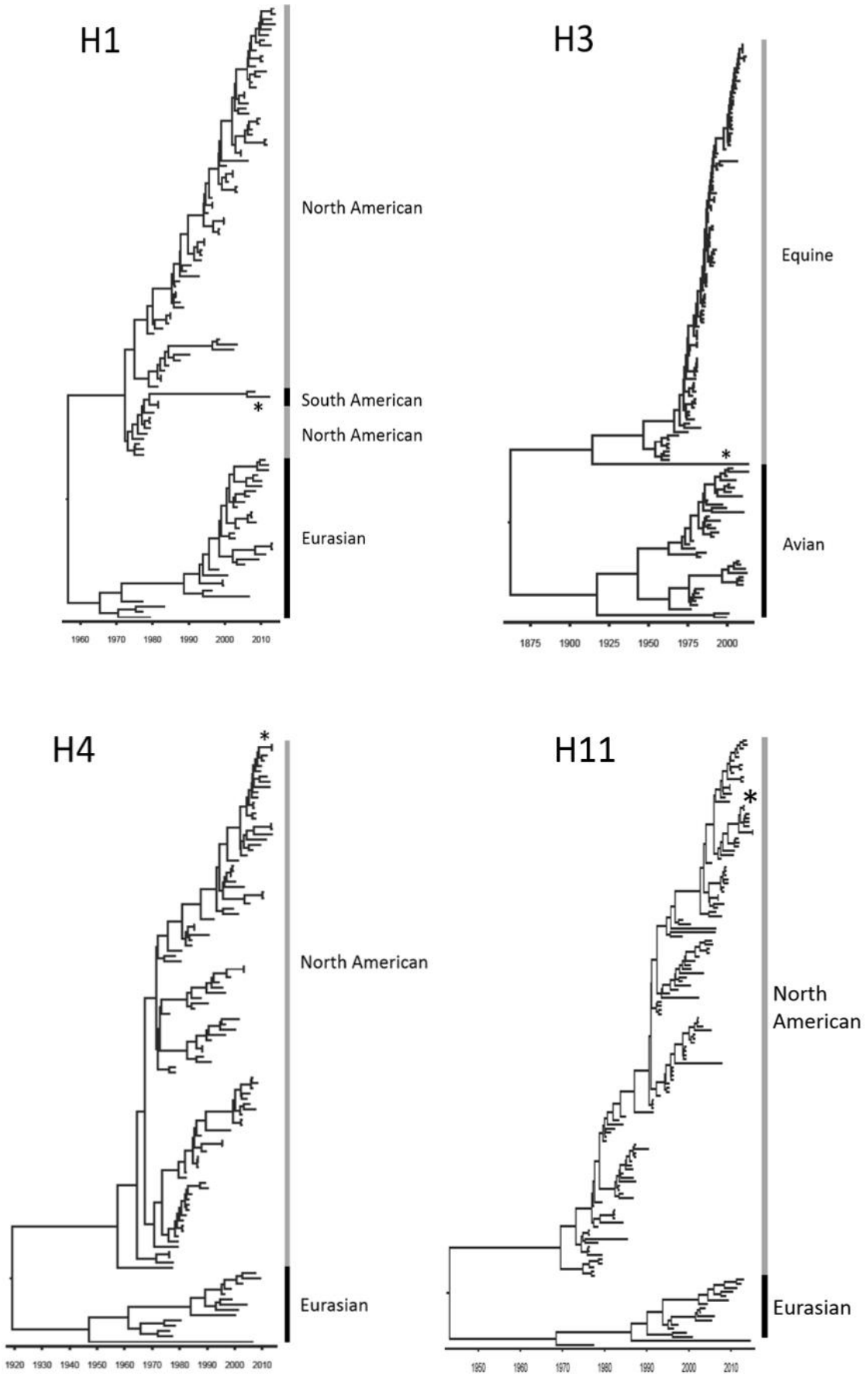
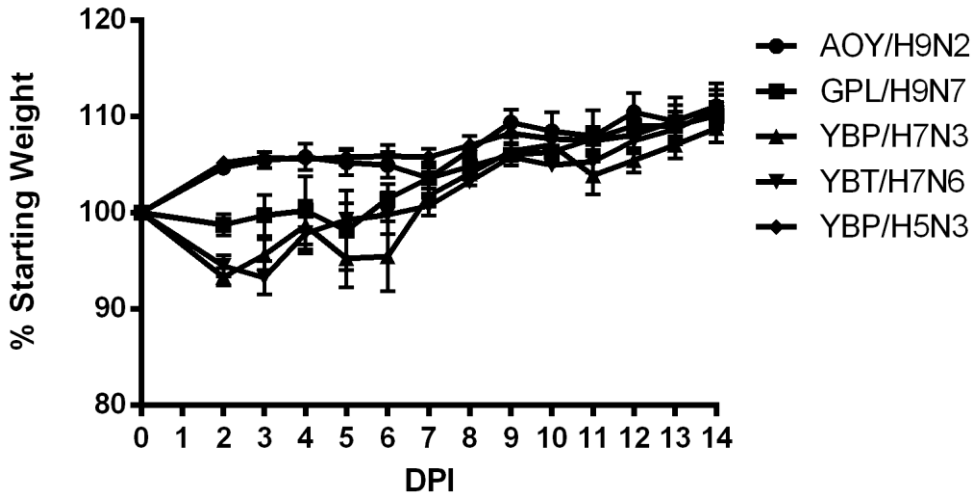

H1
North American
South American
North American
Eurasian
1960 1970 1980 1990 2000 2010
H3
Equine
Avian
1875 1900 1925 1950 1975 2000
H4
North American
Eurasian
1920 1930 1940 1950 1960 1970 1980 1990 2000 2010
H11
North American
Eurasian
1950 1960 1970 1980 1990 2000 2010

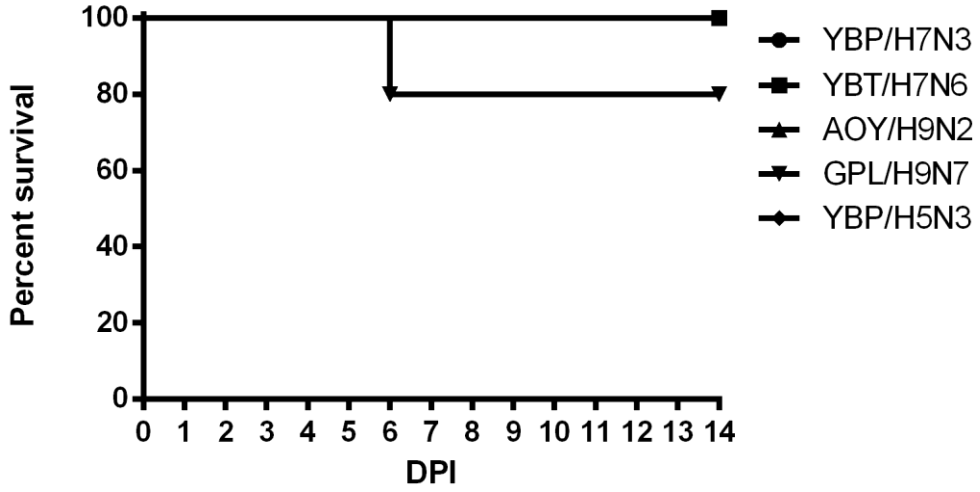
Table 5 Deduced amino acid sequence of the HA cleavage of isolates. Single arginine indicated in bold.

Strain name	Subtype	Cleavage site
A/Yellow-billed pintail/Chile/1/2012	H1N1	PSIQA**R**/GLF
A/Red-fronted coot/Chile/5/2013	H3N6	PEKQT**R**/GLF
A/Yellow-billed teal/Chile/C918/2015	H4N2	PEKAT**R**/GLF
A/Mallard/Chile/C948/2015	H4N2	PEKAT**R**/GLF
A/Yellow-billed pintail/Chile/6/2014	H4N6	PEKAT**R**/GLF
A/Yellow-billed pintail/Chile/7/2014	H4N6	PEKAT**R**/GLF
A/Yellow-billed pintail/Chile/C1267/2015	H5N3	PQRET**R**/GLF
A/Yellow-billed pintail/Chile/10/2014	H7N3	PEKPKT**R**/GLF
A/Yellow-billed pintail/Chile/11/2014	H7N3	PEKPKT**R**/GLF
A/Yellow-billed teal/Chile/12/2014	H7N3	PEKPKT**R**/GLF
A/Yellow-billed teal/Chile/8/2013	H7N6	PEKPKT**R**/GLF
A/Yellow-billed teal/Chile/9/2013	H7N6	PEKPKT**R**/GLF
A/American oystercatcher/Chile/C1307/2015	H9N2	PAASG**R**/GLF
A/Grey plover/Chile/C1313/2015	H9N7	PAASG**R**/GLF
A/Black-necked stilt/1/2013	H11N9	PAIAT**R**/GLF
A/Black-necked stilt/2/2013	H11N9	PAIAT**R**/GLF

Figure 5 Pathogenicity of H5, H7 and H9 viruses *in vivo*. 6 to 8 week old female Balb/c mice (n=11) where intranasally infected with 10^4 $TCID_{50}$ of challenge viruses **(A).** Survival curve until day 14 post infection **(B).**

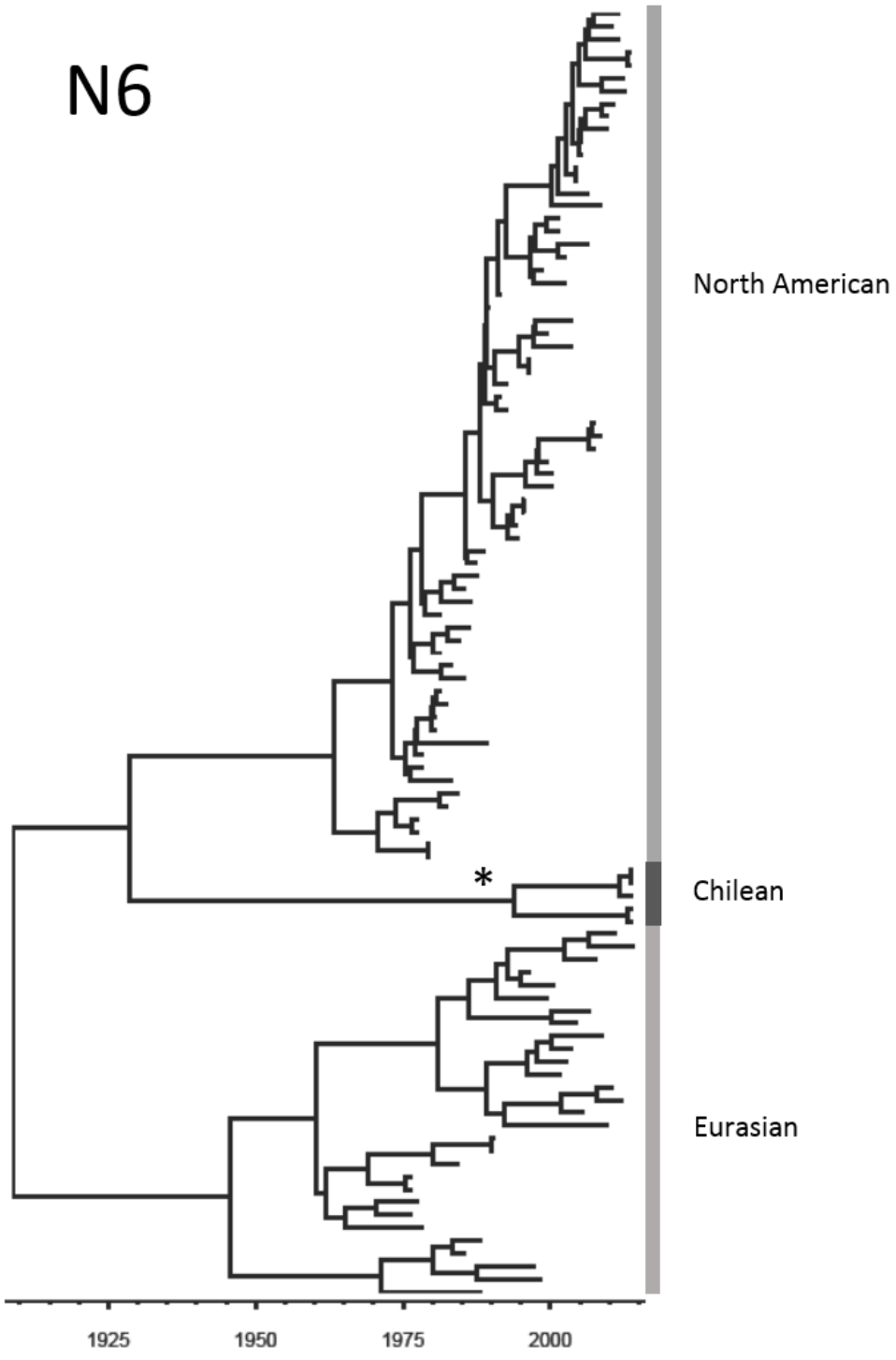

% Starting Weight
120
110
100
90
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DPI
AOY/H9N2
GPL/H9N7
YBP/H7N3
YBT/H7N6
YBP/H5N3

A


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DPI
YBP/H7N3
YBT/H7N6
AOY/H9N2
GPL/H9N7
YBP/H5N3

B

Supplementary Figure S1 Maximum clade credibility tree of the N6 segment. Sequences obtained in this study were compared to South American, North American and Eurasian sequences of avian origin. (*) indicates sequences obtained during this study.


N6
North American
Chilean
Eurasian
*
1925
1950
1975
2000

Chapter 5

CONCLUSIONS

The story of AIV outbreaks in the South American continent is recent and relatively uneventful. In 2002, Chile became the first and only country in South America to date where confirmed isolates of HPAI have been found, occurring at an industrial broiler chicken farm (1). This outbreak was preceded by the detection a month earlier of a highly similar LPAI in turkeys in a nearby farm (2). Phylogenetic analysis of a H7N3 LPAI AIV obtained a year earlier in Bolivia from a wild duck, suggested a common relative between both isolates and supports the hypothesis that the virus in Chile was introduced by wild birds (1,3). Even when these findings emphasize the need to monitor domestic and wild bird species, it is not clear how prevalent AIVs are in South America. Lack of AIV surveillance impacts on the low subtype diversity found so far in South America. This may be due to the fact that the surveillance activities are typically reactive to disease outbreaks or limited to temporary research projects (4). The low sampling effort can be explained by the absence of proof of avian to human transmission of AIV or by niche models that show that AIV prevalence is much higher in wild bird populations in the northern hemisphere than in the southern hemisphere (5,6). Furthermore, Chile and Colombia overlap with all migratory routes that connect North and South America (Pacific, Central, Mississippi and Atlantic flyways) and in theory AIV should be just as prevalent there as it is in the northern hemisphere (7). However, AIV surveillance along the southern hemisphere section of these flyways has been limited and AIV detection and subtype diversity is lower than in the norther hemisphere (8). As a consequence, the introduction or emergence of potentially pathogenic AIVs in the resident avian population of South America cannot be ruled out. This issue becomes of particular concern after the introduction of HPAI H5N8 AIV of Eurasian origin into the wild bird population of North America in late 2014 (9).

This study offers for the first estimation of AIV prevalence in backyard poultry in Chile, the first isolation of AIV from a LAM in South America, the first detection of AIV in wetlands in Chile as well as for the identification of novel clades of AIV hemagglutinins and neuraminidases that are circulating in South America. The isolation of AIV from a LAM in Colombia is significant, particularly due to the large amount of different animal species congregated in close contact. These birds come from populations that are distant from each other and thus have their own ecology. It is this combination of a constant supply of animals, many species in close contact, and multiple sources of birds that make LAMs potential reservoirs for AIV introduction into humans (10). Furthermore, the detection of a novel H5 clade in wild birds in Chile is of particular importance. The H5 subtype is responsible for several outbreaks of AIV throughout Asia and North America, as well as being the cause for fatalities in humans (11,12). The introduction of a novel clade into the HA5 constellation of AIVs therefore poses interesting questions of its potential role as a threat to both animal and human health, despite the LPAI nature of the particular strain isolated in Chile. We also demonstrated that AIV can be found in several different ecosystems throughout South America, ranging from man-made systems (backyard poultry farming, LAM) to shorelines, estuaries, inland wetland and high altitude plains.

There is a clear gap of knowledge regarding AIV in the poultry sector, in particular regarding backyard poultry farming, which by definition result in the most exposed form of poultry rearing to transmission of pathogens from wild birds (13,14). According to our results obtained in Chile by rRT-PCR in backyard poultry (as described in chapter 2), the overall prevalence of AIV between 2012 and 2014 was of 3.8%, compared to 2.6% in Colombia between 2010 and 2012 and 0% in Argentina and Brazil between 1998 and 2005 and between 2006 and 2007, respectively (although AIV in Argentina was confirmed by ELISA) (3,15,16). These discrepancies, between

studies carried out by independent research groups, like in Chile and Colombia, compared to government-led surveillance efforts in South America, could also be due to political reasons, since AIV is of great public concern. The detection and subsequent notification of AIV without immediate HPAI outbreaks could be seen therefore as an unnecessary nuisance.

In March 2015, field sampling in Colombia yielded a prevalence of only 0.67% (n=750) from wild birds and 0% (n=128) from backyard poultry. The 5 positive wild bird samples yielded no subtype information and we were unable to isolate any virus. Details from these samples would have been particularly useful, since it would have allowed us to draw parallels between the subtypes obtained at the LAM and the ones currently circulating elsewhere in the country. In general, our AIV detection efforts in wild birds and backyard poultry in Colombia found a lower incidence than previously reported (15). This difference could be explained due to the date sampling was carried out. March is considered the last month of the dry season in Colombia and one of the driest months of the year. Previous research performed in Colombia established a significant difference between the likeliness of samples being AIV positive during the Colombian rainy season (April-October, 6.9%), than to those samples obtained during the dry season (November-March, 2.9%) (15). This is a clear example of the drawback of performing just one sampling session, since no trends in prevalence can be achieved, potentially leading to false assumptions about pathogen circulation. On the other hand, AIVs circulating at a LAM is a completely new topic in Latin America, since this is the first report of AIV isolation in these kinds of settings.

Animal studies performed for this research thesis indicate that isolates obtained in Colombia and in Chile do not pose an immediate threat to mice (as described in chapters 3 and 4). In general, viruses show to be avian adapted, produce little to moderate morbidity in mice and

replicate poorly in mammalian cells. The only exception to this are the Chilean H11N9 isolates obtained from shorebirds in 2013. These viruses did produce severe weight loss in mice and replicated up to 6 dpi in mice lungs, but interestingly only bound to α2, 3-scialic acid receptors in a receptor binding essay. They did however not transmit efficiently between poultry. Even though it's limited potential to infect poultry, these results are of particular importance. The coastal area of central Chile, where the H11N9 viruses were obtained, is home to several commercial poultry farms that supply the local and international market (14). These findings suggest that an increased surveillance program of shorebirds should be implemented in Chile. Likewise, as described in Chapter 3, the Colombian H11N2 viruses isolated at the LAM in Medellin, even though produced no disease chicken, did transmit efficiently between them as assessed by rRT-PCR at the LAM during the outbreak. This also warrants increased AIV surveillance in LAMs in South America, since continuous circulation of LPAI strains in poultry can give rise to HPAI strains by the insertion of multiple basic amino acid into de HA cleavage site (17–19). Avian influenza isolates with known potential to infect mammals (20,21), other than the described H5, H7, H9 subtypes, were also obtained from wild birds in Chile, as discussed in Chapter 4. Studies have shown that LPAI isolates can replicate without adaptation and to high titers in mouse models causing only sub-clinical disease with limited seroconversion (22). AIVs from wild birds in South America could be constantly crossing species barriers, providing opportunities for the emergence of novel subtypes in unexpected hosts (22). Even though we did not establish the pathogenic potential of all obtained isolates, this should be further explored in other models to better understand mechanisms of AIV infection in mammals.

It has been previously suggested that South America harbors lineages of AIV that are different to others described in the northern hemisphere (8,23). This theory can be further

confirmed by this study, both by analyzing internal and external protein coding segments of the obtained viruses. Internal segments are particularly useful in order to understand the extent of these clades since there are more sequences of South American origin that can be used for phylogenetic analysis, compared to the limited number of hemagglutinins and neuraminidase available in GenBank. These segments show a sharp interhemispheric divide, where the North American and South American lineages are unequivocally different form each other as shown in chapter 3 and 4.

Phylogeny of several viruses obtained during this study support different origins of some isolates. Particularly, all segments of the Colombian H11N2 and Chilean H11N9 viruses are form North American origin. Partial reassortants occur mostly in genes PB1 and NS, where North American lineage segments are found in otherwise South American H1N1, H5N3 and H7N6 viruses. Nevertheless, phylogenetic analysis of the data shows a higher rate of reassortment of surface proteins compared to internal genes. This could be due to: 1) immune selection forced upon the virus by their natural hosts in the presence of a relatively conserved pool of internal genes or 2) to an abundant, but yet unassessed, antigenically diverse availability of surface proteins. The availability of a large pool of dissimilar surface proteins can therefore be associated with a high prevalence of AIV in host species, like ducks and shorebirds (order *Anseriformes* and *Charadriiformes*, respectively). Coincidentally, most of the discovered hemagglutinin and neuraminidase subtypes during this work have been isolated form these order of birds. However, the fact that an atypical H3 has been obtained only from a coot (order *Gruiformes*), may indicate that AIVs circulating in Chilean birds other than *Anseriformes* and *Charadriiformes* are made up of a smaller pool of subtypes. The obtained H3 hemagglutinin for example, is very closely related to the so-called equine 2 influenza viruses and could be either related to their ancestor strain or be a close relative to these viruses circulating in birds due to cross-species transmission of AIV (24).

To date, no other avian equine-like hemagglutinin of South American origin have been submitted to NCBI, and this one in particular could fill an important gap in knowledge regarding the origin of the equine H3N8 pandemic of the early 60's (25).

A relatively rare cross-species transmission rate could be responsible for gene pool segregation, like the one occurring during the increased seasonal prevalence of AIV in wild birds during late summer and fall in Chile, as described in Chapter 4. This could also be driven by desynchronized seasonal peaks of AIV in different host species, hence favoring the seclusion of particular subtype combinations. The presence of a segregated gene pool was also proposed in Chapter 2. The only hemagglutinin subtype identified in backyard poultry flocks in Chile was H12, a subtype not seen in wild birds during this study. This may be due to the population isolation that is subjected upon domestic animals due to limited animal movement and trade.

Internal genes show a North and South American split, but are closer related to a North American super-clade than to Eurasian lineages. An evident interhemispheric divide could indicate that, despite stochastic bottle neck events, native bird species in South America are able to maintain a large antigenic diversity of AIVs. This could happen since a large enough meta-population of hosts can select for antigenically novel viruses and is therefore able to maintain this diversity. Therefore, this AIV diversity has been able to spread efficiently through different hosts and has given rise to viruses of an overall divergent background. Host species behavior, like migration patterns, therefore evidently play a major role in the maintenance and spread of AIV in South America. For example, in Chile the blue-winged teal (*Anas discors*) has only been registered a handful of times, and only in the most northern part of the country (26). This species has been credited as a major player in the interhemispheric spread of avian influenza (27–29), but is unlikely to be able to explain the AIV diversity in Chile. It does, however, most probably play an important

role in Colombia and Central America, where so far only North American-like viruses have been recovered, including the ones obtained in this study at the LAM in Medellin (as described in chapter 3) (15,29). Nevertheless, our studies suggest that the yellow-billed teal (*Anas flavirostris*) and the yellow-billed pintail (*Anas georgica*) are more important in the maintenance of AIV south of the Amazonian basin, as most of the isolates obtained in Chile come from these two species. It is interesting to note that the northern range of the yellow-billed pintail does overlap with the one of the blue-winged teal (30–32). Some populations of birds could therefore be carrying a reassorted virus back to Chile. Other species, like the cinnamon teal (*Anas cyanoptera*) and the yellow-billed teal, could be serving as a bridge between both sides of the Andes, since these two are believed to cross the Andes frequently for wintering and breeding (31,33). However, there are no studies to date that show the actual movement of South American *anatidae* within their range in order to know whether these bird populations are actually responsible for southward movement of viruses. According to AIV surveillance studies performed west of the Andes, samples recovered there are much more likely to be composed of South American lineage segments (23,34,35). Chile (including the Andean Altiplano region) could therefore be an intermediate area, where both lineages frequently intermix. This hypothesis is also supported by the characterization of an H7N3 virus obtained from the Bolivian Altiplano in 2001, that was formed by South- and North American origin gene segments (1). Interestingly, a recent study reporting surveillance data from Peru showed that out of 31 isolated AIVs only one of the them contained South American-like sequences (36). The far-north of Chile and the Altiplano plateau could therefore be the northern most limit of the South American-like AIV influence on the western portion of South America. Further studies should focus on sampling southwards, from southern Colombia, trough Ecuador and Peru to northern Chile to prove this hypothesis, as previously proposed by Ramey *et al*. (27).

Likewise, it is also possible that shorebirds, not ducks, play a major role in the introduction of North American-like gene segments into South America. Based on the genetic similarity between the Colombian and Chilean H11 viruses (as described in chapter 3), we suggest that it is possible for shorebirds to carry AIVs from North America up to the Southern Cone without major reassortment events. Coincidentally, genetic analysis of previously isolated AIVs from shorebirds in Peru, Chile and Brazil are composed exclusively of North American-like gene segments (36–38).

Understanding the extent of viral movement between hemispheres becomes particularly important due to the recent and devastating outbreaks of HPAI H5Nx in domestic poultry and turkeys in North America originated form wild birds and the potential spillover into southern latitudes through bird migration (39). If a highly pathogenic strain established itself in the local bird population of South America, this information would be invaluable. Such an introduction seems plausible, particularly since over 30% of sequences obtained in this study are of North American origin (as described in chapter 4). However, the reason why gene segments of North American lineage are so prevalent in the genetic landscape of AIV in Chile, but not vice-versa, is still unknown (23,27). By analogy, the far south of Chile should see a greater influence of South American AIVs, since the Andes do not represent a physical barrier far south due to lower altitude of the mountains. In fact, as shown by our study, the farther south, the more prevalent the South American clade becomes in the gene ensembles of AIV. Hence, it is possible that the evolutionary divergence of AIVs increases further south, aided by the spatial and climatological segregation of Southern Patagonia that encompasses both southern Chile and Argentina. For example, of the sequences recovered in Arica (latitude 18°29′S), 37% (7 out of 19) of the genes segments are North

American-like, by contrast, approximately 1200 miles south, in Llolleo (latitude 33°35′36″S), only 20% (9 out of 44) of the gene segments obtained are North American-like.

To our knowledge, this is one of the most diverse collections of AIV isolates obtained so far in South America and the data obtained from this research is a valuable contribution to the still poorly understood ecology of AIV in the region. This study attempts to shed light upon the question whether AIVs of South American origin play a major role in the epidemiology and ecology of AIVs in the American Continent. In order to further address these questions, it is undoubtedly necessary to increase sampling efforts throughout the region, both in wild birds as in poultry. In the future, full genome sequence of all isolates obtained in the region is also direly necessary in order to establish phylogenic relationships between them and to study gene exchange rates between both hemispheres. Finally, risk assessment studies of novel South American isolates are important in order to gage the public health and animal welfare impact of these viruses.

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