Risk based surveillance of influenza A virus in wild birds in Chile

C HAMILTON-WEST[1]*, S RUIZ, N BRAVO-VASQUEZ[1], P GALDAMES[1], S GONZALEZ[1], G BOLDT[1],
P JIMENEZ BLUHM[2], S SCHULTZ-CHERRY[2]
[1]Epidemiology Unit Faculty of Veterinary Science, University of Chile, Chile; [2]Infectious Diseases Department,
St. Jude Children's Research Hospital, United States of America

*christopher.hamilton@veterinaria.uchile.cl

Abstract
The aim of this study is to gain epidemiological knowledge on AIV circulating in Chile, following a risk-based approach, all known sites recognised as wild birds concentration areas in Chile were characterised by species diversity, number of interhemispheric migratory species, number of resident species, and species already recognised as reservoirs of AIV. With this information, a risk score was created to orientate the surveillance in high-risk areas for influenza. For the first surveillance season (September 2015 – March 2016), samples have been taken monthly or bimonthly, from 12 sites from north and central Chile. To date 9,747 samples have been collected from September 2015 to March 2016. Positive results have been obtained from all selected sites, resulting in a global prevalence of 2.04%. Complementarily, subtypes H1NX, H1N1, H2N2, H2NX, H3NX, H4N2, H5N3, H5NX, H6NX, H7NX, H8NX, H9N2, H9N7, H9NX, H10NX, H11NX, H12NX, H13NX, H16NX have been identified.

These results reveal the epidemiological importance that Chile could have in the persistence and evolution of AIV in the American continent, and highlights the importance to improve and maintain a risk based AIV surveillance in wild birds in Chile and South America.

Keywords: *Wild birds, disease risk, animal health, preparedness.*

Introduction
Avian influenza viruses are probably one of the main threats for poultry health, and also major concern for the human health, considering the capacity of avian influenza viruses to cause disease in human beings.

Wild birds, especially those related to aquatic environments, are considered as the main reservoirs of influenza virus type A, with the ability to spread the virus among continents when they migrate. This fact has resulted in several research projects worldwide to improve the knowledge on the avian species that can be considered avian influenza reservoirs, ant to identify the diversity, ecology and properties of avian influenza viruses (AIV). Nevertheless, still very little is known about the prevalence and diversity of AIV in South America. The first isolation of AIV from wild birds in this region was made in 2001, during a surveillance program conducted around Lake Titicaca in Bolivia (1). To date, AIV has only been isolated in Chile in 2002, 2008 and 2009 (1, 2), in Peru between 2007 and 2009 (3), Argentina in 2007 and 2008 (4) and 2012 in Colombia (5).

Chile has several wetlands and suitable sites for local and migratory wild birds concentration, considering its more than 5,000 km of coast. In these sites yearly thousands of wild birds migrating from the northern hemisphere during the winter season, following the migratory routes that connect North and South America (Pacific, Atlantic and Mississippi flyways), arrives for nesting and feeding, with the consequent risk for introduction of AIV and for potential reassorment with local strains.

The aim of this study is to gain epidemiological knowledge on AIV circulating in Chile, following a risk-based approach. For this, all known sites recognised as wild birds concentration areas in Chile were characterised and those with the higher risk scores were sampled monthly or bimonthly. To date 9,747 samples have been collected from September 2015 to March 2016 and 4,232 of them were analysed. Positive results have been obtained from all selected sites, resulting in a global prevalence of 2.04%%. Complementarily, subtypes subtypes H1NX, H1N1, H2N2, H2NX, H3NX, H4N2, H5N3, H5NX, H6NX, H7NX, H8NX, H9N2, H9N7, H9NX, H10NX, H11NX, H12NX, H13NX, H16NX have been identified.

These results reveal the epidemiological importance that Chile could have in the persistence and evolution of AIV in the American continent, and highlights the importance to improve and maintain a risk based AIV surveillance in wild birds in Chile and South America.

Materials and methods
Surveillance sites selection
A risk based approach was used. For this, all known sites recognised as wild birds concentration areas in Chile were characterised by species diversity, number of inter hemispheric migratory species, number of resident species, and species already recognised as reservoirs of AIV. For each variable four categories were created (by the median and Q1 and Q3). Then each variable was weighted by researchers knowledge (10%, 10%, 10% and 70%, respectively). With this information, a risk score was calculated for each site to orientate the surveillance in high-risk areas for AIV.

Twelve sites were selected, 10 in the central zone of Chile (where the industrial poultry and swine industry is located) and two sites in the northern area, where presumably the first stop of migratory wild birds could occur.

Sampling and influenza virus detection

From each site, environmental samples of fresh feces were collected, to allow the identification of at least one positive sample assuming a prevalence of 1.5% at each site (6)

Sample collection was done using single-use sterile swabs and placed in tubes containing 1ml Universal Transport Media, UTM™ (Copan Italia S.P.A). Samples were kept at 4ºC for a maximum of four days, then stored at -80ºC until analysis.

RNA extraction was performed at Faculty of Veterinary Science of University of Chile, Chile or at St. Jude Children's Hospital, in Memphis, TN, USA, using Trizol LS Reagent following manufacturer's instructions (Invitrogen). Purified RNAs were protected by adding RiboLock (Life Technologies) and then stored at –80ºC until amplification. RNA was amplified using real time reverse transcriptase PCR (rRT-PCR) in Mx3000P™ Stratagene (Agilent Technologies). Specific primers and probe were used in order to detect influenza A virus matrix gene as described by CDC (2009). The reaction mixture consisted of 3µL of RNA, 5µL of TaqMan Fast Virus 1-Step Master Mix (4x) (Life Technologies), 0.6µL of each Inf A forward and reverse primers, 0.4µL Inf A probe and 10.4µL of RNAase free molecular grade water for a 20µL reaction. The thermal profile included a 50ºC reverse transcription cycle during five minutes, a 95ºC cycle for AmpliTaq® Fast DNA Polymerase UP activation during 20 seconds and 40 amplification cycles (95ºC during three seconds and 60ºC during 30 seconds). Samples under 38 threshold value (Ct) were considered positive.

Virus isolation was attempted on all samples with a Ct. <35 in embryonated chicken eggs as previously described (5). Isolates were confirmed by hemagglutination assay (HA) and rRT-PCR. 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 (7,8). 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 (9).

Isolation and sequencing were done at St. Jude Children's Hospital, in Memphis, TN, USA

Results

In the study period, 9,747 samples were collected and screened for influenza A virus. Positive samples were identified in all surveillance sites, and a global prevalence of AIV of 2.04% was estimated, with a maximum of 2.69 during March 2016 (Table 1).

Table 1. Monthly prevalence of influenza A virus in different surveillance sites in Chile, between September 2015 and March 2016.

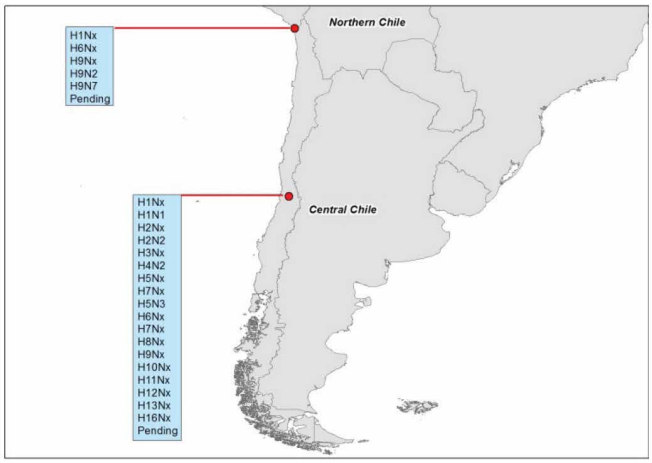
Surveillance site	Month							
	Sep-15	Oct-15	Nov-15	Dec-15	Jan-16	Feb-16	Mar-16	Total
1	4.67	0.78	0.88	1.90	7.29	0.0	0.0	2.22
2	4.00	NA	4.69	NA	0.52	NA	1.04	2.56
3	3.33	1.94	0.0*	0.66	0.0	0.52	2.60	1.29
4	0.28	0.0*	NA	0.00	NA	2.08	NA	0.59
5	NA	0.0*	NA	2.65	NA	6.25	0.0	2.22
6	1.64	NA	NA	8.61	NA	7.25	1.04	4.64
7	2.13	0.00	0.0*	0.0*	0.0	0.52	5.10	1.11
8	0.0	NA	0.0*	NA	1.56	NA	0.52	0.52
9	5.15	0.0*	NA	0.47	NA	1.57	0.26	1.49
10	1.67	NA	0.00	NA	1.56	NA	14.29	4.38
11	1.00	NA	NA	NA	NA	NA	NA	NA
12	1.36	0.0*	NA	2.65	NA	1.04	2.08	1.43
Total	**2.29**	**0.39**	**0.93**	**2.12**	**1.82**	**2.41**	**2.69**	**2.04**

NA: Month without sampling

*: If AIV is present, would be at a prevalence lower than 1.5%

Forty-three virus isolates have been obtained so far, identifying the following subtypes, distributed in the northern and central zone of Chile H1NX, H1N1, H2N2, H2NX, H3NX, H4N2, H5N3, H5NX, H6NX, H7NX, H8NX, H9N2, H9N7, H9NX, H10NX, H11NX, H12NX, H13NX, H16NX (Figure 1). Is important to mention than subtyping of several isolates and positive samples is still on going.

Figure 1. Influenza A virus subtypes identified during September 2015 and March 2016, in northern and central Chile.



Discussion

The improvement in the efforts to understand the ecology of AIVs in wild birds in Chile, including information on AIV prevalence and diversity, could be considered key elements to assess their risk to both humans and poultry populations.

Also, this study provides the largest and most diverse collection of avian influenza (AIV) sequences obtained from wild birds in Chile, and in South America to date. Our results differ from previous research where just three subtypes were identified in three years of surveillance (2007 to 2009) (2). Probably the study design, considering the risk-based approach improved the chance to find the presence of influenza viruses in Chile. Previously to our study, only 14 different HA and NA subtype combinations were described in South America. In contrast, the North American Atlantic Flyway, Europe and Asia, have 94, 91 and 84 unique combinations, respectively (8). These surveillance efforts can provide an early warning message of hazards for public and animal health, and could contribute to the advance on the development of preventive measures in order to protect poultry industry from IAV.

These results reveal the epidemiological importance that Chile could have in the persistence and evolution of AIV in the American continent, and highlights the importance to improve and maintain a risk based AIV surveillance in wild birds in Chile and South America.

References

1. **Spackman E, McCracken K, Winker K, Swayne D.** *Journal of virology* 80, 7760–4, 2006
2. **Mathieu C, Moreno V, Pedersen J, Jeria J, Agredo M, Gutiérrez C, García A, Vásquez M, Avalos P, Retamal**. *Virus research*. 2015.
3. **Williams R, Segovia-Hinostroza K, Ghersi B, Gonzaga V, Peterson T, Montgomery J.** *Journal of wildlife diseases* 48, 910–7, 2012
4. **Alvarez P, Mattiello R, Rivailler P, Pereda A, Davis C, Boado L, D'Ambrosio E, Aguirre S, Espinosa C, Torre J, Donis R, Mattion N.** *Virology* 396, 76–84, 2010
5. **Pereda A, Uhart M, Perez A, Zaccagnini M, Sala L, Decarre J, Goijman A, Solari L, Suarez R, Craig M, Vagnozzi A, Rimondi A, König G, Terrera M, Kaloghlian A, Song H, Sorrell E, Perez D.** *Virology* 378, 363–70, 2008
6. **Salman MD.** *Animal Disease Surveillance and Survey Systems, Methods and Applications*. Blackwell Publishing, 2003
7. **Karlsson E, Ciuoderis K, Freiden P, Seufzer B, Jones J, Johnson J, Parra R, Gongora A, Cardenas D, Barajas D, Osorio J, Schultz-Cherry S.** *Emerging Microbes & Infections* 2, e20, 2013
8. **Olson S, Parmley J, Soos C, Gilbert M, Latorre-Margalef N, Hall J, Hansbro P, Leighton F, Munster V, Joly D.** *PloS one* 9, e90826, 2013
9. **Gonzalez-Reiche A, Perez D.** *Avian Diseases* 56, 1025-1033, 2012