



Circulation of influenza in backyard productive systems in central Chile and evidence of spillover from wild birds

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ABSTRACT

Backyard productive systems (BPS) are recognized as the most common form of animal production in the world. However, BPS frequently exhibit inherent biosecurity deficiencies, and could play a major role in the epidemiology of animal diseases and zoonoses. The aim of this study was to determine if influenza A viruses (IAV) were prevalent in backyard poultry and swine BPS in central Chile. Through active surveillance in Valparaíso and Metropolitan regions from 2012–2014, we found that influenza virus positivity by real-time RT-PCR (qRT-PCR) ranged from 0% during winter 2012–45.8% during fall 2014 at the farm level. We also obtained an H12 hemagglutinin (HA) sequence of wild bird origin from a domestic Muscovy duck (*Cairina moschata*), indicating spillover from wild birds into backyard poultry populations. Furthermore, a one-year sampling effort in 113 BPS in the Libertador Bernardo O'Higgins (LGB ÓHiggins) region showed that 12.6% of poultry and 2.4% of swine were positive for IAV by enzyme-linked immunosorbent assay (ELISA), indicative of previous exposure of farm animals to IAV. This study highlights the need for improved IAV surveillance in backyard populations given the close interaction between domestic animals, wild birds and people in these farms, particularly in an understudied region, like South America.

1. Introduction

Influenza A viruses (IAV) continue to cause diseases outbreaks in animals, including humans, and birds worldwide (Morens et al., 2013). Close interactions between human, swine, and wild and domestic bird viruses can lead to zoonotic spillover events, and subsequently the generation of novel viral strains through periodic exchanges of viral genes (Daszak et al., 2000; Neumann et al., 2009). Poultry production practices, trade of poultry, poultry products and spillover from wild birds have all been recognized as pathways by which avian influenza virus can spread locally and worldwide (Karesh et al., 2005; Gilbert et al., 2006; Kilpatrick et al., 2006; van den Berg, 2009).

Smallholder production, i.e. the many diverse forms of production including backyard productive systems (BPS), is practiced by most rural households throughout the world and presents a major IAV risk (FAO, 2010). BPS are typically defined as smallholder production farms, breeding less than 100 poultry and 10 pigs. These farms are generally maintained for sustenance farming and occasionally generate revenues

from animals or product sales. Most concerning, they generally have poor to absent biosecurity and are often located at the interface between wild and domestic animals making them prime locations for zoonotic and reverse zoonotic transmission of diseases (FAO, 2008; Smith and Dunipace, 2011; Hamilton-West et al., 2012). Indeed, the majority of BPS owners employ limited biosecurity measures and have limited knowledge of animal diseases; therefore, sick animals may be handled, sold, slaughtered and consumed without considering the risk to human health (Iqbal, 2009; FAO, 2010).

In Chile, there are two different realities in term of poultry and swine production. Commercial and industrial farming are very industrialized, operating both breeding and processing units with high biosecurity standards. Some of the bigger companies have even achieved a total integration of the production chain, by integrating food production, breeding and slaughter in one company (APA-ASOHUEVO, 2006; Hamilton-West et al., 2012). Moreover, live animal markets in Chile are uncommon and slaughtering of animals outside a certified facility is prohibited by law. However, in rural areas it is still possible to

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find slaughtering of animals in small size backyard breeding farms for household consumption of eggs and meat. Little is known about IAV in BPS in areas where IAV surveillance is lacking, such as South America (Butler, 2012). Backyard production systems in Chile represent more than 150,000 farmers, raising more than 3.7 million poultry and more than 400,000 pigs (Hamilton-West et al., 2012). Reports from other South American countries are limited to a few seroprevalence studies done in Argentina (Buscaglia et al., 2007), Ecuador (Hernandez-Divers et al., 2006), and Peru (Tinoco et al., 2015). Previous reports of IAV in BPS in Chile have been performed only during short seasons or at limited locations (Bravo-Vasquez et al., 2016; Bravo-Vasquez et al., 2017). Briefly, these studies demonstrated that IAV prevalence, as detected by qRT-PCR, was 27% of infected BPS in an area around a wetland during fall 2014 (Bravo-Vasquez et al., 2016). Seropositivity of backyard swine at a BPS level in this same study ranged between 42% and 60% in spring 2013 and fall 2014. Another study detected 32% seropositivity in backyard swine, but did not report IAV prevalence in poultry (Bravo-Vasquez et al., 2017). Therefore, the aim of this study was to determine the IAV prevalence in backyard poultry and swine in central Chile.

2. Materials and methods

2.1. 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, by the Chilean National Commission for Technological Research (CONICYT), and by the St Jude Children's Research Hospital Institutional Animal Care and Use Committee (IACUC).

2.2. Study area and sample size

This study was carried out in central Chile, including the Valparaiso, Metropolitan and Libertador General Bernardo O'Higgins (LGB O'Higgins) regions. BPS were defined as rural households having up to 100 poultry (Hamilton-West et al., 2012) and up to 10 swine. Only BPS registered in a government subsidized development program were invited to participate in this study. Data presented in this study was gathered from two complementary active surveillance studies, carried out in adjacent locations and at overlapping time points.

Sampling included all poultry species and pigs present in the farm. A minimum of 5 poultry and 5 swine samples per farm were collected as described below. In cases where there were less than 5 animals on the farm, all animals were sampled. Due to lack of information regarding the influenza virus prevalence within BPS in South America and by estimating a high prevalence of IAV at the BPS if the animals would get infected, we assumed a prevalence $\geq 40\%$ and 95% confidence to determine sample size, in order to identify at least one positive animal in each farm. The number of animals present in the farm and sensitivity and specificity of diagnostic tests were considered to adjust the sample size according to formula Eqs. (1) and (2) (Salman, 2003).

$$n = \frac{\log(1-c)}{[\log\{Sp(1-p) + (1-Se)p\}]} \quad (1)$$

Where,

n = Sample size

c = Desired confidence level

p = Disease prevalence

Se = Diagnostic test sensitivity Sp = diagnostic test specificity

$$n_c = \frac{n}{1 + \frac{n}{N}} \quad (2)$$

Where,

n_c = Corrected sample size

n = Sample size obtained with formula 1

N = Population (animals in the backyard farm)

2.3. Sampling and samples analysis

Cloacal and tracheal swabs were collected from poultry in BPS in the Valparaiso and Metropolitan regions throughout four seasons: winter (June–July) 2012; summer (March) 2013; spring (October–November) 2013; and fall (April) 2014) using disposable sterile swabs and stored in cryovials containing 1 mL Universal Transport Media, UTM™ (Copan Italia S.P.A). Samples were kept at 4 °C during sampling and stored at –80 °C until analysis. RNA extraction and real-time RT-PCR (qRT-PCR) analysis were performed at St. Jude Children's Research Hospital, Memphis, TN, USA as described (Karlsson et al., 2013). Briefly, viral RNA extraction was performed on 50 μ L of swab sample on a Kingfisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, USA) using the Ambion MagMax-96 AI/ND viral isolation kit (Life Technologies Corporation, Grand Island, NY, USA). Sample screening was done by qRT-PCR (Bio-Rad CFX96 Real-Time PCR detection System) with the TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA). Specific primer/probes for the influenza matrix gene were used for the qRT-PCR reaction as described (WHO, 2009). Samples with a cycle threshold value (Ct) ≤ 38 were considered positive (Shu et al., 2011) and viral isolation on all samples with Ct ≤ 35 embryonated chicken eggs was attempted in as described (Lira et al., 2010).

2.4. Sequencing

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 elsewhere (Hoffmann et al., 2001). Further sequencing was performed by Sanger sequencing at the University of Wisconsin-Madison Biotechnology Center. Sequence is available under GenBank accession number KX101133.

2.5. Phylogenetic analysis

All publicly available avian H12 sequences at the Influenza Virus Resource (IVR) at NCBI greater than 1500 base pairs were used (n = 174). Duplicated sequences were removed prior to the phylogenetic analysis. BEAST version 1.8.2 was used for the analysis (Drummond et al., 2005; Drummond et al., 2006; Drummond et al., 2012). An 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 generations, sampled every 15000 generations. We then combined the output after removing burn-in (typically 10–20% of sampled chain). Twelve thousand estimated trees and parameters were summarized. 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.

2.6. Seroprevalence

During summer (December–March) 2012 and autumn (Abril–May) 2013, blood was collected from BPS located in LGB O'Higgins Region. Briefly, 1–3 and 3–5 mL of blood were collected from the brachial vein of each bird and from the marginal ear vein of each pig, respectively into a 6 mL vacutainer tube. Samples were kept at 4 °C during transport, centrifuged at 1300 g for 10 min and then stored at –20 °C until

Table 1
Active surveillance in BPS by qRT-PCR.

Season	County	Number of sampled BPS	Number of sampled animals					IAV positive chicken samples by qRT-PCR		IAV positive BPS by qRT-PCR		
			Chicken	Duck	Turkey	Goose	Pig	N	%	N	(%)	CI (%)
Winter 2012	Talagante	16	290	14	1	0	0	0	–	0	–	–
	El Monte	9	229	14	0	1	26	0	–	0	–	–
	Peñaflor	6	156	0	0	0	6	0	–	0	–	–
	Total	31	675	28	1	1	32	0	–	0	–	–
Summer 2013	Talagante	14	283	0	0	0	0	2	0.7	2	14.3	0–32.6
	El Monte	8	299	0	0	0	0	1	0.3	1	12.5	0–35.4
	Peñaflor	10	278	0	0	0	0	3	1.1	3	33.3	1.6–58.
	Melipilla	1	41	0	0	0	0	0	–	0	–	–
	Total	33	901	0	0	0	0	6	0.67	6	18.2	5.0–31.3
Spring 2013	Talagante	9	163	22	2	6	0	0	–	0	–	–
	Peñaflor	9	160	20	1	1	9	0	–	0	–	–
	Padre Hurtado	8	274	6	0	0	9	0	–	0	–	–
	Melipilla	1	30	4	1	2	3	0	–	0	–	–
	San Antonio	6	244	4	0	0	10	1	0.4	1	16.7	0–46.5
	Total	33	871	56	4	10	31	1	0.1	1	3.0	0–8.8
Fall 2014	Talagante	10	190	10	1	3	0	8	4.2	4	40.0	9.6–70.4
	Padre Hurtado	8	214	13	0	0	4	9	4.2	3	37.5	3.9–71.1
	San Antonio	6	212	1	0	6	0	14	6.6	4	66.7	29.0–100.0
	Total	24	616	24	1	9	4	31	5	11	45.8	25.9–65.8

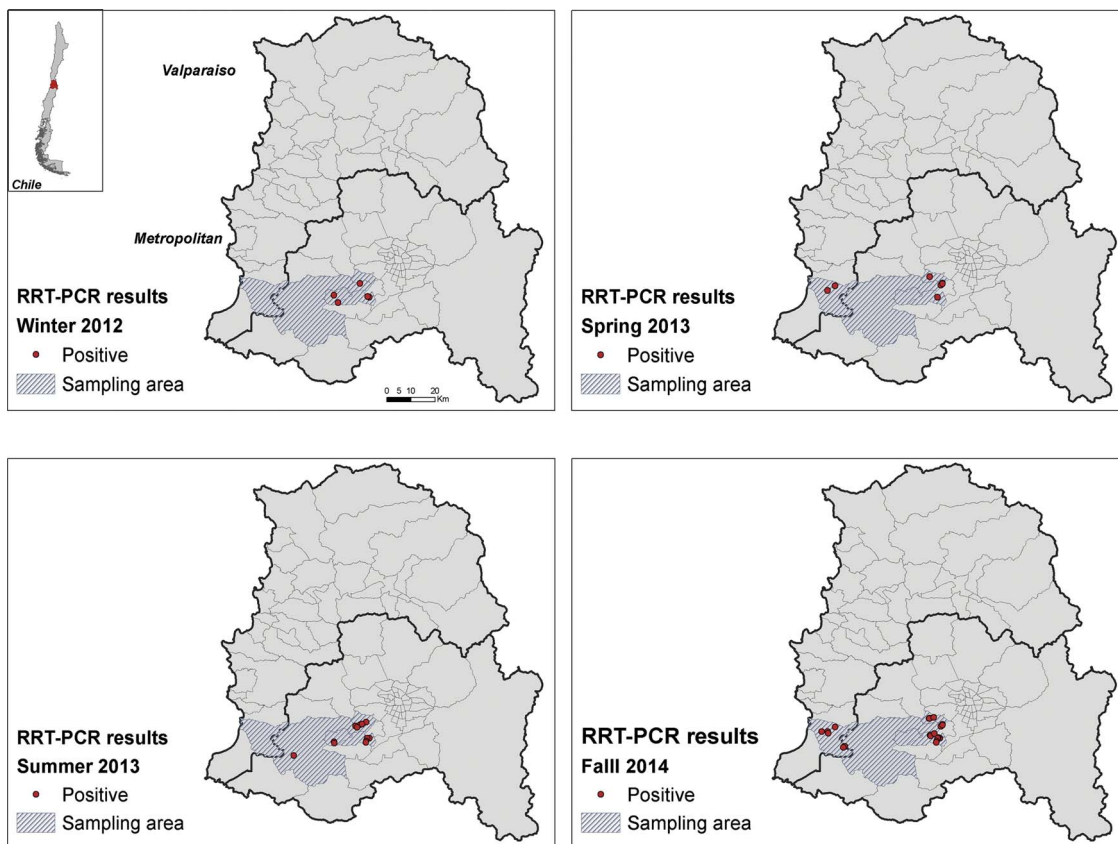


Fig. 1. Spatial distribution of positive BPS for Influenza A by qRT-PCR in Valparaíso and Metropolitan regions.

analysis. Sera was tested for Influenza A by using the IDEXX Influenza A Ab Test (Se: 95.4 and Sp 99.7 for poultry, and Se: 95.3 and Sp: 99.6 for pigs). Plates were read using an IMMUNSKAN Plus (BDSL) microplate reader. No further analysis was conducted on the samples, since remaining sera was sent to the Agricultural and Livestock Service (SAG) of Chile for further analysis as part of their surveillance program.

3. Results

3.1. Influenza a virus is infecting backyard poultry in metropolitan and Valparaíso regions

To monitor for influenza virus from poultry and pigs, 3264 samples were collected from 63 BPS between June 2012 and April 2014. Of these, we obtained 39 (1.2%) positive poultry samples with a Ct value

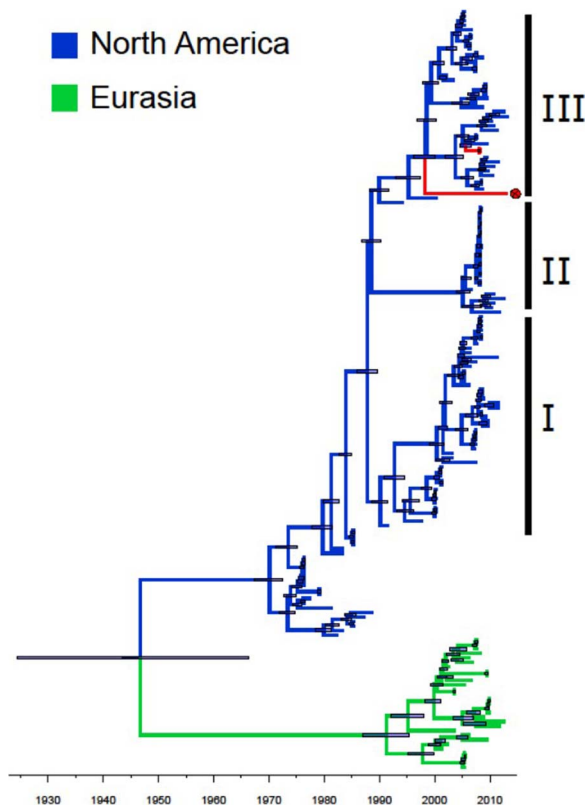


Fig. 2. Phylogenetic relationship between North American and Eurasian H12 segment. Time scaled phylogenetic tree for the H12 segments (n = 174 sequences). In red are represented the other introductions of this subtype into South America (Chile, Peru). The symbol indicates the H12 obtained in Chile. The 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

≤38. Positive Ct values ranged from 27.5 to 37.9 (mean Ct value of 36.8). All positive samples were obtained from chicken, except for one from a domestic duck. The highest percentage of positives were obtained during April (fall) 2014 (5%, 31 out of 616). No positive samples were identified between June and July (winter) 2012 (Table 1). Our attempts to isolate 3 samples with Ct values ≤35 by blind passaging them through embryonated eggs, were unsuccessful.

At the BPS level, positivity ranged from 0% during June–July 2012–45.8% (11 out of 24) in April 2014 (Fig. 1 and Table 1). Intermediate values were obtained in spring (November) 2013 (3%, 1 out of 33) and summer (March) 2013 (18%, 6 out of 33). No positive samples by qRT-PCR were obtained from pigs in this study.

Table 2
Influenza seroprevalence in LBG O'Higgins BPS.

County	Sampled BPS (N)	Number of sampled animals					IAV seropositive BPS (poultry samples)			IAV seropositive BPS (swine samples)		
		Chicken	Duck	Turkey	Geese	Pig	N	%	CI	N	%	CI
Placilla	20	99	6	3	0	35	0	–	–	1	5.6	0–15.6
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	4.0	0–14.2	1	4.0	0–12.2
Litueche	5	12	0	1	0	10	1	20.0	0–47.5	0	–	–
Las Cabras	15	53	7	1	1	9	3	19.0	0–38.9	0	–	–
Paredones	12	35	1	7	0	8	4	32.0	2.9–57.4	0	–	–
Chimbarongo	6	27	4	0	0	6	0	–	–	0	–	–
Pichilemu	19	83	4	0	0	11	5	25.6	3.5–45.9	0	–	–
Marchihue	5	8	8	5	0	5	0	–	–	0	–	–
TOTAL	113	440	38	25	6	127	14	12.6	6.4–18.1	2	2.4	0–5.4

3.2. Identification of a wild bird origin hemagglutinin

While we were unsuccessful at isolating viruses from positive samples, we did obtain one full H12 HA sequence from a domestic Muscovy duck (*Cairina moschata*) cloacal swab. The closest BLAST hit to our sequence was an H12 virus obtained in 2003 from Alberta (A/pintail/Alberta/49/2003) with a 94% sequence identity. Bayesian analysis on the full coding sequence of all available H12 sequences, suggests that there are three H12 lineages currently circulating in wild birds in North America; clades I, II and III (Fig. 2). Our sequence, A/Muscovy duck/Chile/3/2013, belongs to clade III. The 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) (Fig. 2). The results of this analysis therefore indicate the wild bird origin of this strain circulating in BPS in Chile.

3.3. BPS in LBG O'Higgins region of central Chile are seropositive for influenza A virus

A total of 509 poultry sera samples were obtained from animals of different species including domestic chickens (*Gallus domesticus*), ducks (*Anas platyrhynchos*, *Cairina moschata*), turkeys (*Meleagris gallopavo*) and geese (*Anser anser domesticus*) from 113 BPS (Table 2). Only chickens were seropositive with 18 positive samples from 14 BPS making the positivity rate 12.6% at a BPS level. Seropositive birds were identified in BPS located in Navidad, Litueche, Las Cabras, Paredones and Pichilemu counties (Fig. 3). Except for Las Cabras and Paredones, where two farms had two positive chicken and one farm three positive chicken, respectively, all other BPS had only one infected chicken per BPS. In terms of swine, a total of 127 swine sera samples were obtained from 89 BPS, of which 2 were seropositive. The seropositivity rate at BPS level was 2.4%. Seropositive pigs were detected in BPS located in the counties of Placilla and Navidad (Fig. 3 and Table 2).

4. Discussion

The presence of IAV in BPS have been well established in many parts of the world (Karesh et al., 2005; Gilbert et al., 2006; Kilpatrick et al., 2006; van den Berg, 2009). Yet, little data is available from South America, especially in backyard animal populations (Butler, 2012). Our studies demonstrate that animals in BPS in Central Chile are clearly exposed to IAV. Both serological and molecular evidence suggests widespread circulation of IAV in backyard populations, with qRT-PCR evidence obtained in 3 out of 4 sampling seasons in poultry, and seroconversion of farm animals (poultry and swine) detected in 60% of sampled counties. We were also able to sequence a full length H12 HA from a domestic duck that is genetically most closely related to wild bird viruses, suggesting a spillover. This is not surprising, considering

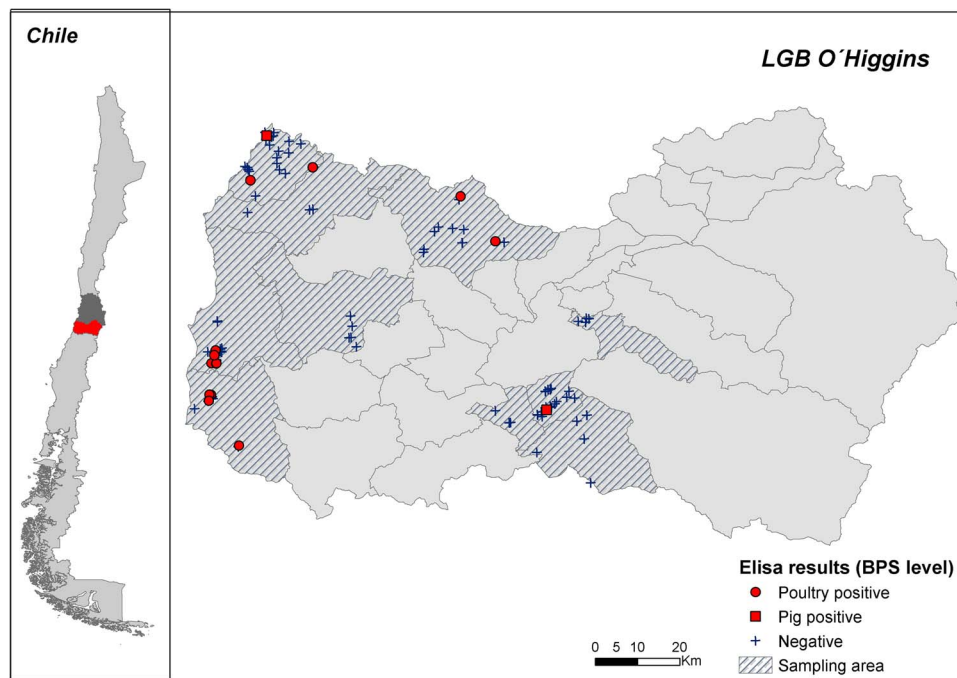


Fig. 3. Spatial distribution of sampled BPS and serology results in LGB ÓHiggins region. Gray area in inset map shows adjacent Valparaíso and Metropolitan regions, red area shows LGB ÓHiggins region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the absence of biosecurity measures implemented at these productive systems and the richness of the Chilean avifauna; Chile is home to more than 51 species of Charadriiforms and 29 species of Anseriformes, both orders of birds known to harbor most of the IAV subtype diversity in nature (Webster et al., 1992; Victoriano et al., 2006; Hamilton-West et al., 2012).

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, Valparaíso region), LPAI H13N2 (2007, wild seagull, Atacama region), LPAI H5N9 (2008, wild seagull, Valparaíso region), LPAI H13N9 (2009, wild gull, Arica and Parínacota region), pH1N1 (2009, domestic turkeys, Valparaíso region) and LPAI H4N8 (2011, domestic turkeys, Valparaíso region) (Mathieu et al., 2010; Pedroni et al., 2012; SAG, 2013; Mathieu et al., 2015). The only exception to wild birds and poultry production sites has been the description of a human-swine reassortant H1N1 virus found in swine a BPS in the Valparaíso region in 2014 (Bravo-Vasquez et al., 2017). By describing a wild bird origin HA in a BPS, our studies show that in Chile these systems can serve as an important link for the transmission and generation of novel IAVs among farm animals, completing the transmission cycle between wild bird habitats and high output breeding farms, as proposed by Suarez, where the Chilean 2002 HPAI outbreak strain was closely related to a LPAI virus obtained earlier from a wild duck at the Titicaca lake in Bolivia (Suarez et al., 2004). Moreover, these findings also suggest that in Chile, these farms could represent a high risk for the transmission of zoonotic IAVs into the human population, particularly due to the close interaction between animals and humans in a low biosecurity environment.

Swine influenza virus (IAV-S) is also recognized as a highly contagious infection of pigs. In Chile, it is one of the most prevalent diseases in large swine production companies, with serological evidence of pH1N1, H1N1 and H3N2 infection (ISP, 2012), and recently described H1N2, H1N1 and H3N2 subtypes (Nelson et al., 2015; Bravo-Vasquez et al., 2017). Even though we were unable to obtain qRT-PCR positive samples of pigs, this might be due to the low number of screened animals by this methodology, only 67 pigs altogether. Our serology data indicates that, even though seropositivity in these pigs seems to be modest, this might also be due to the low sample number obtained, rather than to low IAV-S presence in central Chile.

When comparing our results to other information available in the continent, our results are similar in terms of seropositivity to findings reported in Ecuador, where 11% of seroprevalence of IAV was reported in poultry kept in BPS (Hernandez-Divers et al., 2006). However, when considering positive results for qRT-PCR, positivity levels are higher and increase in summer and fall. This could be due to increased virus load in the environment due to an increase of naïve wild bird hatchlings during summer and after migratory bird have arrived for wintering in the country and are ready for their return to their origin breeding sites (Olson et al., 2014). Our results are consistent with a study conducted in the USA where increased presence of influenza in commercial turkeys in summer and fall was described (Halvorson et al., 1985). Moreover, studies in China have described seasonality in the presentation of avian influenza in fall-winter (Li et al., 2004) while other described it in winter and summer (Park and Glass, 2007). However, our study has several limitations. First, we only sampled BPS systems that participated in a government subsidized development program, hence sampling was not randomized and might not be representative of the overall situation of BPS in central Chile. This may have led to selection bias, resulting in an underestimation of the prevalence of IAV. Second, even though sampling was performed during the four seasons between 2012 and 2014, these sampling efforts were not contiguous, making projections of seasonal patterns based only on this dataset uncertain. Finally, farms screened for seroprevalence were not the same as the farms screened by qRT-PCR, hence no direct association between these two indicators can be established at a BPS level.

5. Conclusions

Influenza A virus is circulating in backyard farms in Chile, representing a risk for both animal and public health. The results of this study highlight the need for improved surveillance of influenza viruses in backyard populations. Poultry and swine production are characterized by a diversity of production systems, with different scales of production, biosecurity measures and characteristics of entry and exit products. Thus, for these production systems to coexist, it is imperative to coordinate efforts to identify risk factors in order to avoid outbreaks that can ultimately affect public health and the economy of the country. It is the authors opinion that BPS should be monitored year-round. There is always a risk of spillover of IAV from wild birds to domestic

animals kept at the BPS, since the presence of a wild bird origin virus at one of these units is unlikely to be an isolated event. We also speculate that BPS in the proximity of irrigation canals, rivers, coastal areas and wetlands, are in higher risk of acquiring IAV from wild bird due to the abundance of waterfowls and shorebirds that could come in close contact to poultry and should therefore be monitored closely for IAV infection. In conclusion, our study shows that IAV is actively circulating in BPS animals in the central region of Chile and that spillover events from wild birds into backyard poultry is a reality. Nevertheless, more studies are needed to understand circulation dynamics of influenza virus in backyard animals in Chile.

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