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Presence of influenza viruses in backyard poultry and swine in El Yali wetland, Chile

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ABSTRACT

In South America little is known regarding influenza virus circulating in backyard poultry and swine populations. Backyard productive systems (BPS) that breed swine and poultry are widely distributed throughout Chile with high density in the central zone, and several BPS are located within the "El Yali" (EY) ecosystem, which is one of the most important wetlands in South America. Here, 130 different wild bird species have been described, of them, at least 22 species migrate yearly from North America for nesting. For this reason, EY is considered as a high-risk zone for avian influenza virus. This study aims to identify if backyard poultry and swine bred in the EY ecosystem have been exposed to influenza A virus and if so, to identify influenza virus subtypes. A biosecurity and handling survey was applied and samples were collected from BPS in two seasons (spring 2013 and fall 2014) for influenza seroprevalence, and in one season (fall 2014) for virus presence. Seroprevalence at BPS level was 42% (95% CI:22–49) during spring 2013 and 60% (95% CI 43–72) in fall 2014. rRT-PCR for the influenza A matrix gene indicated a viral prevalence of 27% (95% CI:14–39) at BPS level in fall 2014. Eight farms (73% of rRT-PCR positive farms) were also positive to the Elisa test at the same time. One BPS was simultaneously positive (rRT-PCR) in multiple species (poultry, swine and geese) and a H1N2 virus was identified from swine, exemplifying the risk that these BPS may pose for generation of novel influenza viruses.

1. Introduction

Wild birds, especially those related to aquatic environments, are considered as main reservoirs of influenza A virus (Webster and Hulse, 2004). Considering that all influenza A virus subtypes have the potential to contribute to the emergence of a pandemic strain through genetic reassorment, and the introduction of an avian or swine influenza virus in the human population may set the stage for influenza pandemic (Webster and Hulse, 2004; Olsen et al., 2006; Van Reeth, 2007). The World Health Organization, Food and Agriculture Organization of the United Nations and the World Organization for Animal Health, advise increasing the global surveillance of influenza virus to improve the preparedness and response to human and animal threats (WHO, 2005).

Chilean poultry and swine production are highly integrated at the industrial level where few companies represent more than 80% of the production, operating with high biosecurity standards (Hamilton-West et al., 2012). Nevertheless, other kinds of animal production are present in Chile, specifically backyard productive systems (BPS) for swine and poultry breeding. BPS are recognized as an important component of small farmers' livelihoods. Poultry species, like chicken, ducks and geese are the more commonly bred animals in BPS, followed by pigs. Usually both, poultry and pigs, do not represent the main economic activity of the farm, but are considered an important contribution to the household's food security and economies' (Randolph et al., 2007). In general, BPS have severe biosecurity deficiencies, which is concerning given that they represent an interface for interactions between domestic species, wild animals and humans (Iqbal, 2009; Conraths et al., 2011).

Given its more than 4000 km of coast, it is not surprising that Chile has several wetlands where thousands of local and wild birds migrating from the northern hemisphere nest and feed each winter. One of the most important wetlands in Chile is the National reserve "El Yali" (EY), located in Valparaíso Region. EY is a natural

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protected area of 520 ha, belonging to the Convention on Wetlands of International Importance especially as waterfowl habitat (Ramsar Convention) (Vilina et al., 2002). The EY ecosystem has a surface bigger than 11.500 ha and 130 different bird species have been described in EY, represented by the orders *Tinamiformes*, *Ciconiiformes*, *Accipitiformes*, *Passeriformes*, *Phoenicopteriformes*, *Falconiformes*, *Strigiformes*, *Galliformes*, *Columbiformes Anseriformes*, *Caprimulgiformes*, *Apodiformes*, *Piciformes, Charadriiformes*, *Gruiformes*, *Pelecaniformes*, *Podicipediformes* and *Sulidiformes*. EY is considered a priority site for influenza A virus introduction (SAG, 2006), since at least 22 are considered inter-hemispheric migratory species. Several BPS are located close to the reserve and within the ecosystem (Hamilton-West et al., 2012) raising concerns about the potential for these systems to be sites for emergence of new influenza strains.

To date, scarce data on influenza A virus in Chile are available. In domestic poultry, H7N3 (A/chicken/Chile/4977/02, GB|AY303634), pH1N1 (A/turkey/Chile/28317-6504-3/2009, GB| GQ866225) and H4N8 have been identified (SAG, 2013). In backyard poultry, there is just one study (Jimenez-Bluhm, unpublished results) which identified an H12NX in a domestic Muscovy duck (*Cairina moschata*) (A/Muscovy duck/Chile/3/2013, GB|KX101133) in central Chile. Therefore, this study aims to identify if backyard poultry and swine bred in BPS in the ecosystem of EY wetland have been exposed to influenza A virus, and if so, to identify viral strains.

2. Materials and methods

2.1. Sampling and samples analysis

The study units were backyard farms located in a radius of 3 km from the EY reserve boundaries. These farms were identified and invited to participate in the study. As BPS, we considered the productive units having up to 100 poultry (Hamilton-West et al., 2012) and up to 50 pigs.

BPS were sampled in two seasons (spring 2013 and fall 2014) for influenza A virus seroprevalence, and in one season (fall 2014) for prevalence. Since there was not information available regarding to influenza viruses within farm prevalence, we assumed a prevalence ≥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 (Salman, 2003). Nevertheless, a minimum of five samples were collected, and in cases when there were less than five animals in the farm, all of them were sampled. Sampling included all poultry species and pigs present in the farm.

Blood was collected from the brachial vein of birds (1–3 mL) and from the marginal ear vein of pigs (3–5 mL) and placed into a 6 mL vaccutainer®. Samples were kept at 4 °C during transportation and serum was obtained by centrifugation at 1,300*g* for 15 min at Faculty of Veterinary Science of University of Chile (FAVET), and then stored at −20 °C until analysis.

To screen for the presence of influenza A virus, pools of cloacal swabs (poultry) and nasal swabs (swine) were collected in tubes containing 1 mL universal viral transport medium (Healthlink®). Samples were collected using disposable sterile swabs, and at least one pool per animal species present in the farm was collected. Each pool consisted in at most 9 swabs from animals of the same species (Ladman et al., 2012), and if more than 9 samples had to be collected, another pool of the same specie was added. Samples were maintained at 4 °C until arrival to FAVET, where the samples were stored at −80 °C until analysis.

Influenza A virus seroprevalence was determined using the ELISA assay (IDEXX Influenza A Ab Test) following manufacturer's instructions (Sensitivity: 95.4% and Specificity 99.7% for poultry, and Se: 95.3% and Sp: 99.6% for pigs) (Idexx, 2016). Plates were read using an INMUNSKAN Plus (BDSL) microplate reader. We considered a BPS positive if at least one sample gave positive results.

RNA extraction was performed using Trizol LS following manufacturer's instructions (Invitrogen). Purified RNAs were protected by adding RiboLock (Life Technologies) and then stored at −80 °C 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 3 μL of RNA, 5 μL of TaqMan Fast Virus 1-Step Master Mix (4x) (Life Technologies), 0.6 μL of each Inf A forward and reverse primers, 0.4 μL Inf A probe and 10.4 μL of RNAase free molecular grade water for a 20 μL reaction. The thermal profile included a 50 °C reverse transcription cycle during 5 min, a 95 °C cycle for AmpliTaq® Fast DNA Polymerase UP activation during 20 s and 40 amplification cycles (95 °C during 3 s and 60 °C during 30 s). Samples under 38 threshold value (Ct) were considered positive. Swine influenza virus subtyping was carried out using H1/H3 and N1 specifics primers and probes assays (Richt et al., 2004; CDC, 2009; Gunson et al., 2010). Subtype was confirmed by Sanger sequencing at St. Jude Children Research Hospital (SJCRH) using segment specific primers as described by Hoffmann et al. (2001). We considered BPS positive if at least one sample gave positive results.

In each BPS, a questionnaire was applied by a semi-structured interview to backyard owners to collect information regarding to animal handling and biosecurity measures.

2.2. Data analysis

A database was built into a Microsoft Excel® spreadsheet which included the information collected in field activities and laboratory results. Firstly, descriptive statistics were provided to characterize the BPS, taking into consideration structure, biosecurity and trade elements. Then influenza A virus prevalence and seroprevalence were estimated. Finally, the independent variables obtained in the survey were tested as risk factors for the presence of antibodies against influenza A virus (ELISA) and influenza A virus (rRT-PCR), by a logistic regression model (Dohoo et al., 2009) using R statistical software (http://www.r-project.org).

3. Results

3.1. BPS characterization

Forty six BPS were identified in the study site, from which 40 agreed to participate in the study. The total number of animals present in these farms was 1798 chickens, 45 turkeys, 34 pigs, 25 geese and 5 ducks. The median animals by farm were 30 domestic chickens (min:6, max:80), 0 turkeys (min:0, max:23), 0 geese (min:0, max:14), 0 ducks (min:0, max:5), and 0 pigs (min: 0, max: 20). Fifty five percent of the owners breed animals for sales to neighbors, family and tourists. The other 45% of the owners primarily keep animals for household consumption. In terms of confinement, 70% of the households keep their animals in a mixed confinement system, i.e., free ranging during the day and confined during night, while 25% are free-range and 5% are permanently confined. When asked how they handled mortalities, the most common answers were that they burnt (43%) or buried (23%) their dead animals, while 13% throw their dead animals in the garbage, or throwing them far away (10%). Three percent of the owners did nothing, leaving the dead animals just as they found them. It is important to mention that 10% of the participants did not want to answer this question.