Molecular identification and phylogenetic characterization of influenza A virus at a wildlife-livestock interface in Mexico

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Abstract

Influenza A virus (IAV) outbreaks constitute a constant threat to public health and pose a remarkable impact on socio-economic systems worldwide. Interactions between wild and domestic birds, humans, and swine can lead to spillover events. Backyard livestock systems in proximity to wetlands represent a high-risk area for viral spread. However, some gaps remain in our knowledge of IAV transmission at the wildlife – livestock interface in Mexico. Hence, the study aimed at molecular identification and phylogenetic characterization of IAV in the wild duck – backyard livestock interface at a wetland of Mexico. A total of 875 animals were tested by real-time RT-PCR (qRT-PCR). We detected IAV in 3.68% of the wild ducks sampled during the winter season 2016 – 2017. Nonetheless, the samples obtained from backyard poultry and swine tested negative. The highest IAV frequency (11.10%) was found in the Mexican duck (Anas diazi). Subtypes H1N1, H3N2, and H5N2 were detected. Phylogenetic analysis of influenza viruses isolated from wild ducks of the Lerma marshes revealed that hemagglutinin (HA) gene sequences were related to waterfowl, swine, and poultry IAV strains previously isolated in the United States and Mexico. In conclusion, the co-circulation of three IAV subtypes in wild ducks close to backyard farms in Mexico, as well as, the local identification of HA gene sequences genetically related to Mexican livestock IAV strains and also to North American waterfowl IAV strains, highlight the importance of the Lerma marshes for influenza surveillance given the close interaction among wild birds, poultry, pigs, and humans.

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Influenza virus at a wildlife-livestock interface

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Summary

Influenza A virus (IAV) outbreaks constitute a constant threat to public health and pose a remarkable impact on socio-economic systems worldwide. Interactions between wild and domestic birds, humans, and swine can lead to spillover events. Backyard livestock systems in proximity to wetlands represent a high-risk area for viral spread. However, some gaps remain in our knowledge of IAV transmission at the wildlife – livestock interface in Mexico. Hence, the study aimed at molecular identification and phylogenetic characterization of IAV in the wild duck – backyard livestock interface at a wetland of Mexico. A total of 875 animals were tested by real-time RT-PCR (qRT-PCR). We detected IAV in 3.68% of the wild ducks sampled during the winter season 2016 – 2017. Nonetheless, the samples obtained from backyard poultry and swine tested negative. The highest IAV frequency (11.10%) was found in the Mexican duck (*Anas diazi*). Subtypes H1N1, H3N2, and H5N2 were detected. Phylogenetic analysis of influenza viruses isolated from wild ducks of the Lerma marshes revealed that hemagglutinin (HA) gene sequences were related to waterfowl, swine, and poultry IAV strains previously isolated in the United States and Mexico. In conclusion, the co-circulation of three IAV subtypes in wild ducks close to backyard farms in Mexico, as well as, the local identification of HA gene sequences genetically related to Mexican livestock IAV strains and also to North American waterfowl IAV strains, highlight the importance of the Lerma marshes for influenza surveillance given the close interaction among wild birds, poultry, pigs, and humans.

Keywords: backyard poultry, backyard swine, hunter-harvested wild ducks, phylogenetic analysis, qRT-PCR, the Lerma marshes.

Introduction

Outbreaks of influenza A viruses (IAV) have caused considerable harm to the animal and human health with several global socio-economic impacts (McLeod, Morgan, Prakash, & Hinrichs, 2005). Wild aquatic birds such as the Anseriformes and Charadriiformes are the major natural reservoirs of IAV, except H17 and H18 subtypes detected in bats (Munster et al., 2007; Tong et al., 2013). In addition to its natural reservoir circulation, IAV can cross the species barrier and infect a wide range of hosts including, canids, equids, humans, poultry, and swine (Joseph, Su, Vijaykrishna, & Smith, 2017).

Wild waterfowl migration has a dominant role in the global influenza virus spread (Franklin et al., 2019; Ren et al., 2016). Every year, low pathogenic avian influenza (LPAI) outbreaks among waterfowl occur during migration when contact rates among waterfowl populations are high (van Dijk et al., 2014). Indeed, migratory wild birds are associated with intra- and intercontinental dissemination of IAV as well as the geographic dispersion of IAV subtypes in wild bird species and poultry residents (Fourment, Darling, & Holmes, 2017; Humphreys et al., 2020).

The wildlife – livestock interface represents a significant source of disease in domestic flocks (Wang, Jiang, Jin, Tan, & Xu, 2013). Some production practices such as free-range farming may increase the likelihood of contact among wild and domestic populations, and represent a critical factor for persistence and transmission of IAV (Cappelle et al., 2014). Likewise, backyard systems are considered suitable areas for influenza surveillance due to the complex interactions between swine, poultry, humans, and wild animals (Hamilton-West et al., 2012; Jimenez-Bluhm et al., 2018).

Previous research has provided some insights into the dynamic of IAV at the wildlife –livestock interface (Wiethoelter, Beltrán-Alcrudo, Kock, & Mor, 2015). Phylogenetic analyses have revealed high sequence homology among IAV circulating in pigs, poultry, and wild birds (Bergervoet et al., 2019; Osbjer et al., 2016). Ecological and phylogeographical analyses have shown that migratory waterfowl, domestic ducks, free-ranging animals, and agroecosystems are crucial in the maintenance and viral spread (Wei, Lin, & Xie,

2015). Serological and molecular studies of IAV have evidenced the viral circulation in backyard farms situated close to "el Yali", one of the most important wetlands in South America (Bravo-Vasquez et al., 2016).

The Lerma marshes constitute a critical surveillance area where wild ducks, backyard poultry, backyard pigs, and humans co-occur in time and space. Some molecular studies in wild ducks and their environment have been conducted in this Natural Protected Area (Cuevas-Dominguez et al., 2009; Ornelas-Eusebio, Obregon-Ascencio, Chavez-Maya, & Garcia-Espinosa, 2015; Ramirez-Martinez, Loza-Rubio, Mosqueda, Gonzalez-Garay, & Garcia-Espinosa, 2018). Nevertheless, the aims of these studies were no centered on the interrelation among diverse species, and a persistent gap in knowledge about IAV dynamics in the wildlife – livestock interface remains in the region. Therefore, this study performed molecular and phylogenetic characterization of IAV in wild ducks, backyard swine, and backyard poultry at this interface of Central Mexico.

Materials and methods

Ethics Statement

All procedures and protocols were approved by the ethical committee "Subcomite Institucional para el Cuidado y Uso de Animales de Experimentacion – SICUAE" (MC-2017/2-5, MC-2017/2-6), Universidad Nacional Autonoma de Mexico (UNAM).

Study area

This study was carried out in the Municipality of Lerma de Villada, State of Mexico (19deg13'–19deg26'N, 99deg22'–99deg34'W). The Lerma marshes provide a suitable habitat for thousands of migratory waterfowl than arrived annually from Canada and the United States in the Mexican Central Plateau on their migratory route to the south (Barragan Severo, Lopez-Lopez, & Babb Stanley, 2002). Animals were sampled from ten different study units: wild ducks from the Lerma marshes, as well as pigs and poultry from nine backyard farms located within a 6 Km radius of the Lerma marshes boundaries (Figure 1).

Sample collection

Wild ducks from the Lerma marshes and the surrounding backyard farms were sampled during the winter and the early spring season 2016 – 2017. Each wild bird species was identified based on the descriptions provided by Howell & Webb (2010) and classified according to Clements et al. (2019). The sample selection was done by a convenience sampling method. Cloacal swabs from 597 hunter-harvested wild ducks, nasal swabs from 175 pigs, as well as cloacal and oropharyngeal swabs from 103 poultry (hens, cocks, ducks, geese, and turkeys) were collected and stored in cryovials containing 1 ml of Brain Heart Infusion (BHI) broth and 1ml of Eagle's Minimum Essential Medium (EMEM) in wild duck/poultry and pig samples, respectively. The biological samples were kept refrigerated at 4 degC during transportation and stored at -80 degC until analysis.

qRT-PCR and viral isolation

All samples were centrifugated at 2500 rpm for 5 minutes (mini centrifuge with A12-2P rotor "Dlab: D2012 plus"). The biological samples were pooled by species and collection date (3 to 4 samples/pool). Subsequently, automated RNA extraction was performed from 200µL of each pooled supernatant using the Cador® Pathogen 96 QIAcube HT Kit (Qiagen) following the manufacturer's instructions. The qRT-PCR was carried out with the VetMAX-Gold SIV Detection Kit (Life Technologies) following the manufacturer's instructions (Ma et al., 2014). Negative and positive controls were included in each run. Samples with a cycle threshold value (Ct) <38 were considered positive as described by the manufacturer. Positive pools were tested again individually. Positive IAV samples were inoculated into the allantoic cavity of 9-to-11-day-old specific-pathogen-free (SPF) chicken embryos as described before (Spackman & Killian, 2014). The allantoic fluid was harvested, and a haemagglutination assay was performed with 0.5% chicken red blood cells (Killian, 2014).

Sequencing

Whole-genome sequencing was performed using an Ion Torrent Personal Genome Machine (PGM) System (Life Technologies), following the manufacturer's specifications. Sequencing libraries were constructed using the Ion Plus Fragment Library (Life Technologies) by physical fragmentation of genome segments with the Bioruptor(r) Sonication System to generate fragments of approximately 200 base pairs (bp). The DNA fragments were linked to Ion-compatible adapters and amplified using the Ion PGMHi-Q OT2 Kit (Life Technologies) on an Ion OneTouch 2 System. The sequencing reaction was carried out using an Ion 314 Chip v2 and the Ion PGM Hi-Q Sequencing Kit (Life Technologies). Reads were aligned with a Q-score [?] 20 and assembled with the AssemblerSPAdes v 5.4.0 program. The average genomic coverage depth of the Ion Torrent PGM was up to 300-fold.

Phylogenetic analysis

The reference sequences used for the phylogenetic analysis were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov). The nucleotide sequence identity selected was greater than 96% using BLAST (Basic Local Alignment Search Tool) from the National Center for Biotechnology-NCBI (NCBI Resource Coordinators et al., 2018). Sequences were aligned using the MUSCLE algorithm (Edgar, 2004). The phylogenetic tree was constructed by the maximum-likelihood method with 1,000 bootstrap replicates in MEGA 7.0.26 (Kumar, Stecher, & Tamura, 2016).

Results

AIV molecular detection

A total of 875 biological samples of wild ducks (n=597), poultry (n=103), and swine (n=175) were collected from November 2016 to May 2017 (Figure 2). Eight dabbling duck species and two diving duck species were sampled for IAV detection by qRT-PCR in the Lerma marshes, State of Mexico. Twenty-two wild dabbling ducks (3.68%) tested positive for IAV (Table 1). The IAV positive samples were obtained from November to February, predominantly in the mid-winter season. The IAV positive wild dabbling ducks belonged to four species: Green-winged Teal (*Anas crecca*), Blue-winged Teal (*Spatula discors*), Northern Shoveler (*Spatula clypeata*) and Mexican duck *(Anas diazi*) (Figure 3). Blue-winged Teal was the most frequent species in the sampling area, followed by Green-winged Teal. The highest IAV frequency was found in Mexican duck, followed by Northern Shoveler (Table 1). All swabs from wild diving ducks, pigs, and poultry tested negative for IAV by qRT-PCR.

Phylogenetic characterization

Nine IAV detected in wild dabbling ducks from the Lerma marshes were isolated in chick embryos. All eight influenza gene segments of each virus were sequenced, and the sequences were submitted into the GenBank database (Table 2).

The phylogenetic analysis of the HA gene showed three major clades. The upper clade had 3 strains (A/blue-winged_teal/EstadodeMexico/Lerma/UIFMVZ507/2017 (H5N2), A/ green-winged_teal/EstadodeMexico/Lerma/UIFMVZ80/2016 (H5N2), and A/northern_-shoveler/EstadodeMexico/Lerma/UIFMVZ613/2017 (H5N2)) related to Mexican swine and chicken sequences of H5N2 viral subtype (Figure 4). Whereas one strain (A/Mexican_-duck/EstadodeMexico/Lerma/UIFMVZ377/2016 (H5N2)) was phylogenetically related to wild duck sequences of H5N1, H5N2, H5N5, and H5N9 subtypes reported in United States of America (Figure 4). The HA gene from A/blue-winged_teal/EstadodeMexico/Lerma/UIFMVZ322/2016 (H1N1) was found in a different clade, and it was strongly genetically related to U.S. and Mexican swine H1N1 viruses (Figure 4). Finally, in the lower clade were found four sequences (A/green-winged_teal/ EstadodeMexico/Lerma/UIFMVZ169/2016 (H3N2), A/green-winged_teal/EstadodeMexico/ Lerma/UIFMVZ456/2017 (H3N2), A/green-winged_teal/EstadodeMexico/Lerma/UIFMVZ 465/2017 (H3N2), and A/blue-winged_-teal/EstadodeMexico/Lerma/UIFMVZ478/2017 (H3N2)) genetically similar to U.S. and Mexican swine H3N2 subtypes (Figure 4).

Discussion

This study describes the molecular identification and phylogenetic characterization of IAV at a wildlife - livestock interface in the Lerma marshes, Mexico. One of the findings in this study was the detection of 22 wild dabbling ducks (3.68%) positive to IAV in the mid-winter. Seasonality, geographical location, and between-year fluctuations can modify the IAV prevalence and subtype distribution. For instance, higher IAV prevalence (>30%) occurs predominantly before and during the fall migration, while lower prevalence (<1%) is related to the spring migration in North America (Lickfett, Clark, Gehring, & Alm, 2018; Wille, Brojer, Lundkvist, & Jarhult, 2018). However, the prevalence of AIVs in their natural hosts also depends on the species (Munster et al., 2007).

Mallards (*Anas platyrhynchos*) and other dabbling duck species (*Anas* spp.) have higher IAV prevalence that diving ducks (Vandegrift, Sokolow, Daszak, & Kilpatrick, 2010). Specie-specific differences in IAV prevalence are related to several factors including intrinsic physiological characteristics, feeding behavior, shedding patterns, population size, and adaptation to the virus (Munster et al., 2007; Papp et al., 2017; J. G. van Dijk, Verhagen, Wille, & Waldenstrom, 2018; Wilcox et al., 2011). During 2016 - 2017, we detected IAV in four wild dabbling duck species from the Lerma marshes: Mexican Duck (11.10%), Northern Shoveler (6.67%), Green-winged Teal (4.97%), and Blue-winged Teal (4.28%). These results are consistent with multiyear surveillance conducted by Ferro et al. (2010), which found a higher IAV prevalence in Blue-winged Teal, Northern Shoveler, and Green-winged Teal in the United States. Likewise, in our study, none of the diving duck species sampled resulted positive to IAV. This result was in line with the research conducted by Munster et al. (2007), which suggested less-efficient virus transmission by diving ducks.

Previous studies have identified IAV in waterfowl from the Lerma marshes (Cuevas-Dominguez et al., 2009; Ramirez-Martinez et al., 2018). IAV subtypes H7N3, H6N2, and H4N2 have been isolated from anatids, sentinel ducks, and wild bird habitats of the State of Mexico (Barron-Rodriguez, Chavez-Maya, Loza-Rubio, & Garcia-Espinosa, 2018; Cuevas-Dominguez et al., 2009; Ornelas-Eusebio et al., 2015). Findings in our study revealed the co-circulation of three subtypes: H5N2, H3N2, and H1N1.

The phylogenetic analysis showed that the HA gene sequence of an H5N2 subtype isolated from a Mexican duck (*A. diazi*) was closely related to migratory wild duck viruses from North America. This result is consistent with the studies conducted by Barron-Rodriguez et al. (2018) and Ornelas-Eusebio et al. (2015), who reported influenza viruses highly similar to North America waterfowls IAV strains.

The phylogenetic characterization also revealed that the HA gene sequences of the H5N2, H3N2, and H1N1 subtypes isolated from Northern Shoveler, Green-winged Teal, and Blue-winged Teal shown to be more phylogenetically related to swine and chicken sequences from Mexico. The characterization of a swine-like or avian-like IAV isolated from wild ducks is not very well documented. Nevertheless, Cuevas-Dominguez et al. (2009) previously reported the isolation of an H7N3 subtype from a wild bird that showed 100% identity with an IAV isolated from a quail of the United States. Similarly, Olsen, Karasin, and Erickson (2003) isolated the H1N2 influenza virus (A/Duck/NorthCarolina/91347/01) in a wild duck that was phylogenetically related to swine-like reassortant H1N2 and H3N2 viruses of the United States of America.

Areas with close interactions between humans, livestock, and wild animals have been considered as "hotspots" for IAV interspecies transmission (Wei et al., 2015). Interspecies transmission of IAV is dependent on several factors, including virus shedding, host immune system, host receptor specificity, virus stability in the environment, and the degree of close contact with different host species (Joseph et al., 2017; Yassine, Lee, Gourapura, & Saif, 2010). The presence of IAV in wild migratory birds, along with the proximity of domestic flocks to surface water has been significantly associated with increased risk of local outbreaks (Karasin, Brown, Carman, & Olsen, 2000; Walsh, Amstislavski, Greene, & Haseeb, 2017). However, in our study, all swine and poultry samples were negative to IAV by qRT- PCR.

Negative IAV results could indicate a possible absence of recent viral exposure at the time of sample collection or lack of viral adaptation to alternate hosts such as poultry and swine (Awosanya, Babalobi, Omilabu, Nguku, & Ogundipe, 2013; Bourret et al., 2017). Likewise, some research has reported zero prevalence in backyard systems during winter or discordant detection of AIV subtypes in time and space between poultry

and wild birds (Jimenez-Bluhm et al., 2018; Verhagen et al., 2017).

In conclusion, this study identified the co-circulation of three different subtypes of IAV in wild ducks close to backyard farms in the Lerma marshes during the winter season 2016 – 2017. Direct interspecies transmission among wild and livestock animals was no determined. Nevertheless, phylogenetic analysis suggests possible genetic relationships among wild birds, swine, and poultry, as well as the transboundary movement of influenza within the American continent most likely associated with the waterfowl migration. Therefore, increased long-term molecular IAV surveillance throughout the year in the Lerma marshes would further contribute to understanding the contribution of backyard animals, migratory, and resident wild ducks in the disease epidemiology.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Data Availability Statement

Data supporting the findings of this study are included in this article. The genome sequences of influenza viruses are available in the NCBI GenBank database.

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Table 1. Influenza A virus frequency in wild ducks from the Lerma marshes, 2016 – 2017.

	Scientific name	**Common name**	**n**	**IAV (%)+**
Dabbling ducks	*Anas acuta*	Northern Pintail	43	0 (0)
	Anas crecca	Green-winged Teal	201	10 (4.97)
	Anas diazi	Mexican duck	9	1 (11.1)
	Mareca americana	American Wigeon	41	0 (0)
	Mareca strepera	Gadwall	47	0 (0)
	Spatula discors	Blue-winged Teal	210	9 (4.28)
	Spatula clypeata	Northern Shoveler	30	2 (6.67)
	Spatula cyanoptera	Cinnamon Teal	10	0 (0)
Diving ducks	*Aythya collaris*	Ring-necked duck	3	0 (0)
	Oxyura jamaicensis	Ruddy duck	3	0 (0)
	Total		**597**	**22 (3.68)**

+ by the qRT-PCR assay. Abbreviation used: IAV, Influenza A virus.

Table 2. NCBI GenBank accession numbers of Influenza A viruses detected in wild ducks from the Lerma marshes.

Influenza A virus+	GenBank accession number
A/Green-winged_-teal/EstadodeMexico/Lerma/UIFMVZ80/2016 (H5N2)	MT325963-MT325970
A/Mexican_-duck/EstadodeMexico/Lerma/UIFMVZ377/2016 (H5N2)	MK828137- MK828144
A/Blue-winged_-teal/EstadodeMexico/Lerma/UIFMVZ507/2017 (H5N2)	MT305332-MT305339
A/Northern_shoveler/EstadodeMexico/Lerma/UIFMVZ613/2017 (H5N2)	MT325927-MT325934
A/Blue-winged_-teal/EstadodeMexico/Lerma/UIFMVZ322/2016 (H1N1)	MT311129-MT311136
A/green-winged_-teal/EstadodeMexico/Lerma/UIFMVZ169/2016 (H3N2)	MT318090-MT318097
A/Green-winged_-teal/EstadodeMexico/Lerma/UIFMVZ456/2017 (H3N2)	MT318132-MT318139
A/Green-winged_-teal/EstadodeMexico/Lerma/UIFMVZ465/2017 (H3N2)	MT318432-MT318439
A/Blue_winged_-teal/EstadodeMexico/Lerma/UIFMVZ478/2017 (H3N2)	MT320087-MT320094

+ The nine IAV isolated in 9-to-11-day-old specific-pathogen-free (SPF) chicken embryos.

Figure 1. Geographical distribution of the backyard farms in Lerma, State of Mexico (n=9). Yellow circles indicate the poultry farms, red circles show poultry and pig farms, the purple circle indicates a pig farm, and the blue line indicates the Lerma marshes boundaries.

Figure 2. Timeline of sample collection in wild ducks, backyard poultry, and backyard pigs. From Lerma, State of Mexico. The black numbers represent the total of sampled animals. The circled numbers indicate the ID numbers of the farms.

Figure 3. Influenza A virus frequency in wild dabbling ducks. The relative percentage of wild ducks positive to IAV by specie and sampling month.

Figure 4. Maximum-likelihood phylogenetic tree of the hemagglutinin (HA) gene segment of Influenza A viruses from wild ducks of Lerma, State of Mexico 2016 – 2017. Mexican viruses isolated are shown in colors by subtype: blue (H5N2), purple (H1N1), and red (H3N2). Scale bars indicate nucleotide substitutions per site.