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MOLECULAR IDENTIFICATION OF AVIAN VIRUSES IN NEOTROPIC CORMORANTS (*PHALACROCORAX BRASILIANUS*) IN CHILE

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ABSTRACT: We identified two RNA (paramyxovirus and coronavirus) and two DNA (adenovirus and herpesvirus) viruses in a common aquatic bird, the Neotropic Cormorant (*Phalacrocorax brasilianus*), and determined their phylogenetic relationships to other global circulating variants. We analyzed 104 cloacal swabs from individuals collected at locations in Central Chile. Sequences were obtained from amplicons using consensus primers targeting conserved genes of the virus families *Paramyxoviridae*, *Coronaviridae*, *Adenoviridae*, and *Herpesviridae*. A total of 20.2% of the samples was positive for coronavirus, 8.7% for adenovirus, and 3.8% for herpesvirus. No paramyxoviruses were detected. All coronaviruses were identified as viruses of the *Gammacoronavirus* genus, closely related to the infectious bronchitis virus clade (bootstrap clade support=75%). All adenovirus samples were identified as *Aviadenovirus*, related to a gull and falcon adenovirus (Bayesian posterior probability=0.86). The herpesviruses identified were related to the infectious laryngotracheitis virus (*Gallid herpesvirus* 1) of the genus *Iltovirus* (bootstrap clade support=99%). We provide information about the diversity of viruses circulating among apparently healthy Neotropic Cormorants.

Key words: Adenovirus, Chile, cormorant, coronavirus, herpesvirus, paramyxovirus, *Phalacrocorax*.

INTRODUCTION

Surveillance of pathogens is a pivotal task for predicting emerging diseases (Christaki 2015). Because most emerging diseases are zoonoses (Jones et al. 2008), pathogen surveillance and discovery in wildlife have rapidly grown using genetic and genomic methods (Blanchong et al. 2016). Aquatic birds are natural reservoirs for a variety of emerging viruses (Czeglédi et al. 2006; Olsen et al. 2006; Kim et al. 2007). Cormorants and shags, for instance, are the main reservoirs of Newcastle disease virus (Leighton and Heckert 2008), an avian paramyxovirus type 1 responsible for causing severe disease in poultry worldwide. Consequently, a variety of epidemiologic and virologic studies have been done with cormorant-associated avian paramyxovirus type 1 infections (Glaser et al. 1999; Rue et al. 2010). Although avian influenza virus (AIV) has been isolated from Great Cormorants (*Phalacrocorax carbo*; Süss et al. 1994; Chen et al. 2006), the low prevalence of AIV antibody may indicate that cormorants are unlikely to be involved in the circulation of AIV (Artois et al. 2002; Pereda et al. 2008; Cross et al. 2013). However, little is known about other viruses infecting cormorants. Coronaviruses have been identified only once in Great Cormorants (Chu et al. 2011), although serosurveys have detected a high prevalence of antibodies against infectious bronchitis virus (IBV) serotypes (Gallo et al. 2013). The same study also identified antibodies against avian adenovirus in a high proportion of both cormorant species analyzed. Similarly, Travis et al. (2006) detected antibodies against avian adenovirus 1 in the Galapagos Cormorant (*Phalacrocorax harrisi*), although no adenovirus has been reported infecting cormorants. The Lake Victoria cormorant virus is a herpesvirus isolated once from a Little Pied Cormorant (*Microcarbo melanoleucos*) from Australia (French et al. 1973).

We report several avian viruses in the Neotropic Cormorant (*Phalacrocorax brasilianus*). The species occurs from Texas, US to southern South America, in a variety of salt, fresh, and brackish water habitats. The

Neotropic Cormorant is a synanthropic species, living in and using human buildings for nesting, but information about viruses infecting this species is absent. Our aim was to identify the presence of RNA (i.e., paramyxovirus and coronavirus) and DNA (i.e., adenovirus and herpesvirus) viruses in Neotropic Cormorants from Chile and to characterize the phylogenetic relationships of these viruses with other circulating variants. Understanding these viruses can help to predict and prevent diseases that may affect wildlife populations, humans, and domestic animals.

MATERIALS AND METHODS

Sample collection

During 2015–16, we obtained 104 cloacal swabs from wild Neotropic Cormorants sampled at a breeding colony in Valdivia (39°41′29″S, 73°11′34″W), from carcasses of adults collected by hunters during hunting season (April to August) in surrounding areas of Chillan (36°32′58″S, 72°05′00″W) and Valdivia (39°51′42″S, 73°17′26″W), and from individuals received at a wildlife rescue center in Valdivia (39°48′6″S, 73°15′20″W; Centro de Rehabilitación de Fauna Silvestre, Universidad Austral de Chile), Chile. Swabs were deposited in universal transport medium, placed in a cryogenic shipper with liquid nitrogen, and transported to the laboratory and stored at –80 C until analyzed. We conducted our study according to the animal welfare guidelines of the Bioethical Committee of the Universidad Austral de Chile and with permissions given by the Chilean Agriculture and Livestock Service (Servicio Agrícola y Ganadero 2010).

Viral gene amplification and sequencing

Viral RNA and DNA were extracted from swabs using QIAamp MinElute virus spin (Qiagen, Valencia, California, USA) and PureLink viral RNA/DNA (Invitrogen, Carlsbad, California, USA) kits according to the manufacturers' instructions. Reverse transcription of the RNA samples and PCR amplification were performed with the SuperScript™ III one-step reverse transcription PCR system and high-fidelity DNA polymerase (Invitrogen), respectively. For paramyxovirus detection, a PCR protocol targeting a segment of 200–500 base pairs (bp) of the L-protein gene of the *Paramyxovirinae* subfamily with the consensus paramyxovirus primers PAR-F1 and PAR-R (Tong et al. 2008) was performed. The PCR conditions of 55 C for 30 min, followed by 2 min at 94 C, were used for complementary DNA synthesis and predenaturation, followed by 40 cycles of 15 s at 94 C, 30 s at 55 C, and 40 s at 68 C, and a final extension of 68 C for 5 min. For the detection of coronaviruses, a set of consensus coronavirus primers (2Bp/4Bm) targeting 250 bp of the RNA-dependent RNA polymerase gene of all groups of coronavirus (Stephensen et al. 1999) were used in the following PCR protocol: 55 C for 30 min and 2 min at 94 C, followed by 40 cycles of 15 s at 94 C, 30 s at 45 C, and 40 s at 68 C, and a final extension of 68 C for 5 min. Adenoviruses were detected using consensus adenovirus primers HexF1 and HexR1 targeting 800 bp of the hexon protein gene of three genera (*Aviadenovirus*, *Siadenovirus*, *Atadenovirus*) of adenoviruses (Mase et al. 2009). A PCR was performed using a SapphireAmp® Fast PCR master mix (Takara Bio, Ohtsu, Japan), with an initial denaturation at 94 C for 5 min followed by 35 cycles 94 C for 30 s, 55 C for 30 s, and 72 C for 30 s, and a final extension of 72 C for 8 min. Finally, for herpesvirus, a nested PCR was performed using consensus herpesvirus primers targeting 170 to 365 bp of the DNA polymerase gene (VanDevanter et al. 1996). The first PCR round included a multiplex panel of three degenerate primers (DFA, ILK, and KG1), whereas the second PCR round included two degenerate primers (TGV and IYG). The PCR conditions were an initial denaturation at 94 C for 5 min, 45 cycles of 94 C for 30 s, 46 C for 60 s, and 72 C for 60 s, followed by a final extension of 72 C for 7 min. Five microliters of product from the first PCR was used as a template for the second PCR. An aliquot of ultrapure sterile water and a corresponding commercial vaccine were included as negative and positive controls, respectively, during all PCR amplifications. All products of the expected size were purified and sequenced in both directions in an ABI 3500 genetic analyzer (AustralOmics, Valdivia, Chile). All sequences from this study were deposited in GenBank (nos. KY769942–KY769951).

Phylogenetic analysis

Sequence data from each virus were edited and assembled using BioEdit (Hall 1999). A data set of each virus family was retrieved from the National Center for Biotechnology Information and used for phylogenetic analysis. Sequence alignments were performed with MAFFT version 7 (Katoh and Standley 2013). Maximum likelihood and Bayesian trees were inferred using PhyML 3.1 (Guindon et al. 2010) and MrBayes v3.2.5 (Huelsenbeck and Ronquist 2001). The best-fit model of nucleotide substitution was identified by JModeltest 2.1 (Darriba et al. 2012). The robustness of individual nodes was assessed with a

bootstrap resampling process of 1,000 replicates for maximum likelihood analyses or an average SD of the split frequencies <0.01 and effective sample size >200 for Bayesian analyses. A pairwise genetic distance matrix of each predicted protein sequence was built with MEGA6 (Tamura et al. 2013) with 1,000 bootstrap replicates to roughly estimate the genetic difference between the sequences obtained here and the corresponding data sets.

RESULTS

Coronavirus-positive reactions were detected in 21 samples (prevalence: 0.202; 95% confidence interval [CI]: 0.13–0.292), adenovirus in eight samples (prevalence: 0.087; 95% CI: 0.04–0.158), and herpesvirus in four samples (prevalence: 0.038; 95% CI: 0.013–0.093) of Neotropic Cormorants (Table 1). No paramyxoviruses were found. Most of the positive samples came from individuals captured in the breeding colony site.

In the phylogenetic analysis, the coronaviruses detected belonged to the *Gammacoronavirus* genus (Fig. 1). All sequenced viruses clustered together in a monophyletic clade with a within-clade genetic diversity of 8.4%±1.9%. The Neotropic Cormorant coronaviruses identified were closely related to the clade containing IBV and Turkey coronavirus strains supported by a bootstrap value of 75% and a Bayesian posterior probability of 0.73. The cormorant clade showed 7.5%±1.8% of genetic distance. Most of the diversity came from the virus in one sample (16094/Neotropic Cormorant) that was 19.8% divergent from the IBV-clade (Supplementary Material Fig. S1). Likewise, the genetic distance with the delta-, beta-, and alpha-coronaviruses was 20.4%±3.9%, 20.4%±3.7%, and 19.7%±3.7%, respectively.

All adenovirus sequences showed a 100% identity and were identified as an *Aviadenovirus* sharing a common ancestor with a Herring Gull (*Larus argentatus*; KC309439) and a Northern Aplomado Falcon (*Falco femoralis*; AY683541) adenovirus supported by a bootstrap value of 60% and a Bayesian posterior probability of 0.8 (Fig. 2). The genetic distance of the cormorant adenovirus was 32.5%±2.4% divergent from other aviadenoviruses, whereas with Sia-, Ata-, and Mast-adenovirus was 57.7%±3%, 60.4%±3%, and 62.4%±3%, respectively (Fig. S2).

The phylogenetic analysis of the herpesvirus sequences revealed that the Neotropic Cormorant herpesviruses were related to Gallid herpesvirus 1 of the *Iltovirus* family with a bootstrap clade support value of 100% and a Bayesian posterior probability of 1 (Fig. 3), showing a genetic distance of 5.3%±1.5% and 2%±1.4% between both clades detected at both nucleotide and amino acid levels, respectively. The herpesviruses found in the Neotropic cormorants were 23.6%±4.2% genetically distant from other Iltoviruses, 39.6%±6.7% distant from *Scutavirus*, 41.9%±6.4% from *Simplexvirus*, 45.8%±6.3% from *Varicellovirus*, and 47.2%±6.4% from *Mardivirus* (Fig. S3).

DISCUSSION

We detected coronaviruses, herpesviruses, and adenoviruses in samples from the Neotropic Cormorant, one of the most abundant marine bird species in the Americas. Surveillance of coronaviruses has led to the discovery of an impressive diversity of novel viruses, mainly alpha- and beta-coronaviruses (Tang et al. 2006; Woo et al. 2012; Anthony et al. 2013). A smaller, but growing, body of information has become available on the role of wild birds in the epidemiology and evolution of coronaviruses, mainly in species of Anseriformes and Charadriiformes (Hughes et al. 2009; Woo et al. 2012; Chen et al. 2013) but also Passeriformes (Woo et al. 2009). Most of these studies have demonstrated that avian hosts harbor a genetically diverse range of gamma- and delta-coronaviruses We found a coronavirus prevalence of 20.2%, relatively high compared with other studies in aquatic wild birds (6.4%, Muradrasoli et al. 2010; 0.95%, Kim and Oem 2014; 12.5% overall, although a 54.2% prevalence was detected in the subsample of Great Cormorants, Chu et al. 2011). Serologic studies have also shown a high prevalence of antibodies against different

TABLE 1. Distribution by site in Chile of cloacal samples obtained in 2015–16 from Neotropic Cormorants (*Phalacrocorax brasilianus*) and tested by PCR for coronavirus, adenovirus, and herpesvirus.

Site	Total	*Coronaviridae*, *N* positive	*Adenoviridae*, *N* positive	*Herpesviridae*, *N* positive
Breeding colony	27	18	7	2
Valdivia area	23	0	1	0
Chillan area	49	1	0	2
Wildlife rehabilitation center	5	2	0	0
Total	104	21	8	4

IBV serotypes in Imperial (53%) and Magellanic (64%) cormorants (*Phalacrocorax atriceps* and *Phalacrocorax magellanicus*, respectively) (Gallo et al. 2013). Those results suggest that cormorants are a relevant player in the maintenance of genetic diversity of coronaviruses. Only viruses of the *Deltacoronavirus* genus have been previously detected infecting Great Cormorants (Chu et al. 2011). Our study complements previous information, adding these potential viruses of *Gammacoronavirus*. All samples clustered together in a clade that shares a common ancestor with IBV clade. The Mass serotype was the dominating IBV strain in Chile until 2008 when it was replaced by the Q1-like strain (Servicio Agrícola y Ganadero 2010) and vaccination using IBV 4/91 began in poultry in Chile. Since the phylogeny was constructed with a relatively conserved genomic segment of the virus, more detailed genetic characterization of the viruses detected including variable genes, such as the S1 spike gene, is needed to determine the potential role of wild aquatic birds, particularly cormorants, as reservoirs of IBV-like strains.

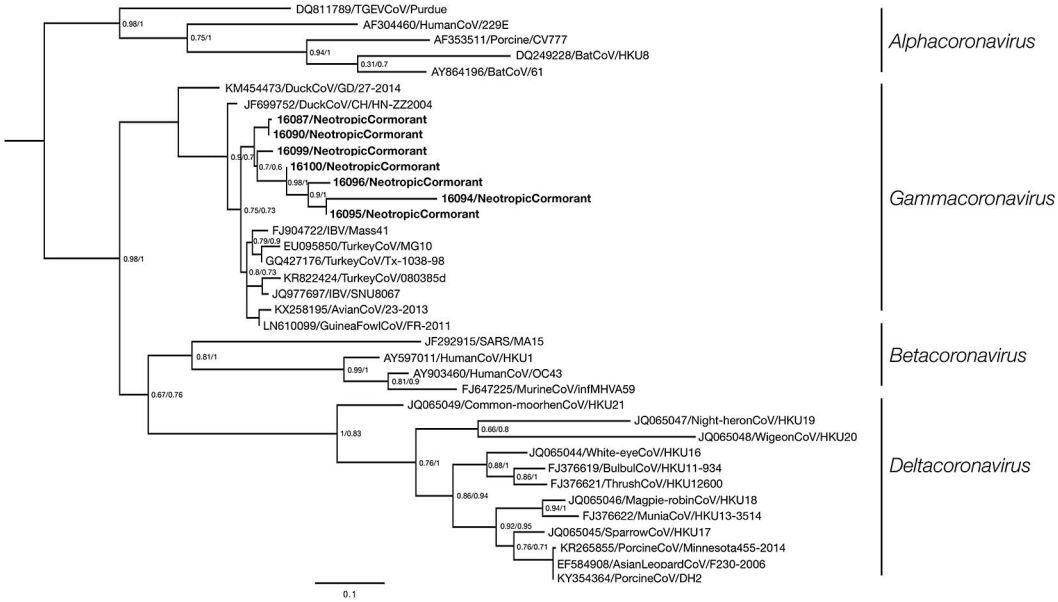

FIGURE 1. Maximum likelihood phylogenetic tree based on the RNA-dependent RNA polymerase gene of coronavirus sequences from PCR of cloacal swabs taken from Neotropic Cormorants (*Phalacrocorax brasilianus*) at several locations in Chile in 2015–17. The Neotropic Cormorant coronavirus is in bold. Alpha-coronaviruses were used as outgroup. The best-fit model of nucleotide substitution was HKY+I+G. Bootstrap and posterior probabilities (from Bayesian analysis) values are represented for each node.

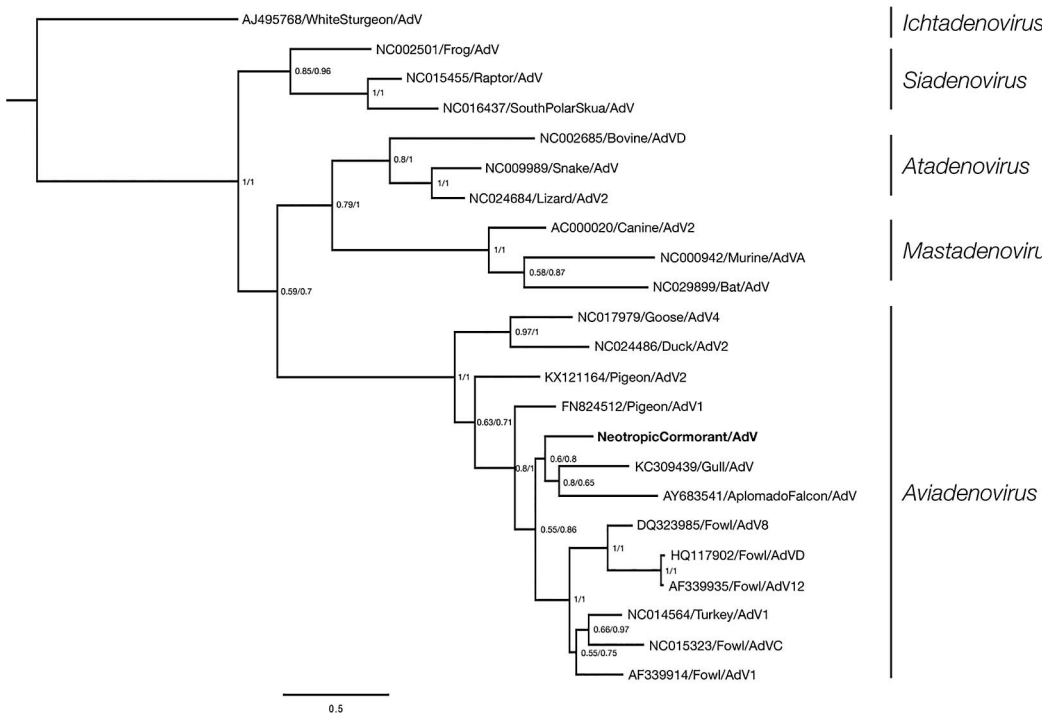

FIGURE 2. Bayesian phylogenetic tree based on the hexon gene of adenovirus sequences from PCR of cloacal swabs taken from Neotropic Cormorants (*Phalacrocorax brasilianus*) at several locations in Chile in 2015–17. The Neotropic Cormorant adenovirus is in bold. The white sturgeon (*Acipenser transmontanus*) adenovirus (AJ495768) was used as outgroup. The best-fit model of nucleotide substitution was GTR+I+G. Bootstrap (from maximum likelihood analysis) and posterior probabilities values are represented for each node.

Adenoviruses are distributed worldwide and are often asymptomatic, causing infections mostly in immunocompromised animals. The prevalence of adenoviruses we detected was 8.7%. Although there is evidence of avian adenovirus 1 antibodies in Galapagos (Travis et al. 2006), Imperial, and Magellanic (Gallo et al. 2013) cormorants, no virus has been detected in cormorants. The sequence information we provided is the first identifying an adenovirus in cormorants. All cormorant adenoviruses produced sequences with 100% identity, and clustered with sequences of the genus *Aviadenovirus*. The genetic distance from the clade containing poultry adenoviruses (i.e., fowl and turkey adenoviruses) was 36.7%. The most phylogenetically related viruses were a gull adenovirus (34% of genetic distance) and an Aplomado Falcon adenovirus (34.7%), which suggested that this virus in Neotropic Cormorants could not have originated from poultry by spillover of fowl aviadenovirus infection. The gull adenovirus was detected from virus-associated lesions in the cloacal bursa of a Herring Gull and a Lesser Black-backed Gull (*Larus fuscus*) during an outbreak of circovirus infection (Bodewes et al. 2013). The falcon adenovirus was isolated during an outbreak affecting several falcon species (Schrenzel et al. 2005). This evidence suggests that this clade of adenovirus could include viruses that are potentially virulent for avian hosts.

A herpesvirus (Lake Victoria cormorant virus) was previously described infecting a nestling Little Pied Cormorant (French et al. 1973). However, no further findings have been published about this virus, and no genetic information is available to compare phylogenetically with other sequences. Our study provided the first sequence information of a herpesvirus infecting a cormorant species.

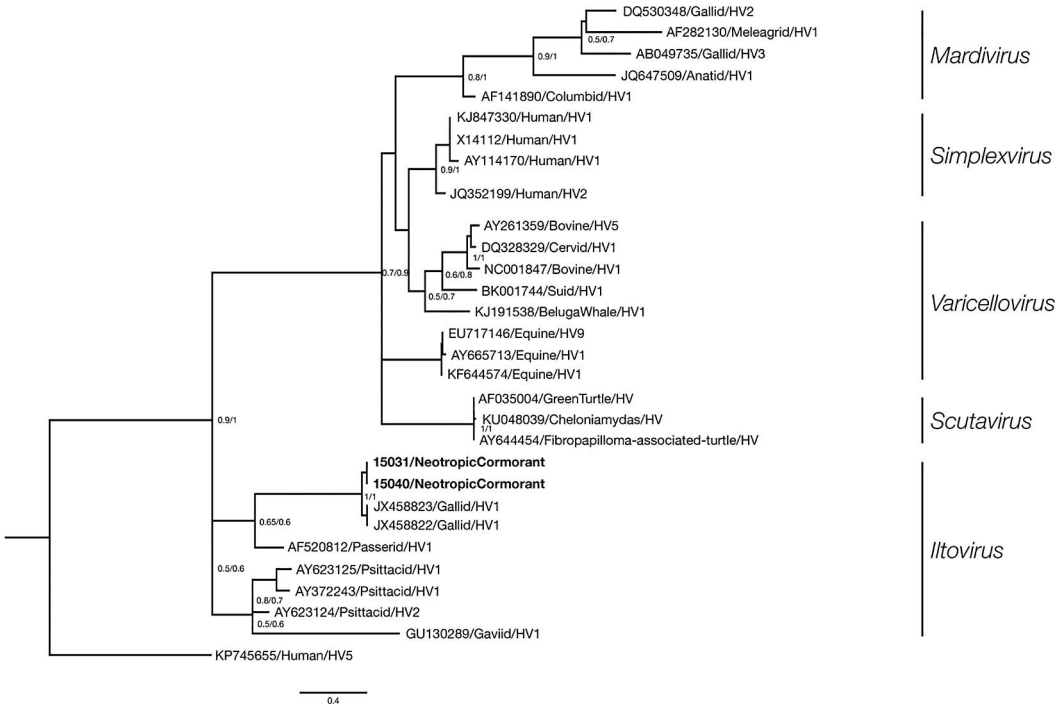

FIGURE 3. Maximum likelihood phylogenetic tree based on the DNA polymerase gene of alpha-herpesvirus sequences from PCR of cloacal swabs taken from Neotropic Cormorants (*Phalacrocorax brasilianus*) at several locations in Chile in 2015–17. The Neotropic Cormorant herpesviruses are in bold. The human herpesvirus HV-5 (KP745655) was used as outgroup. The best-fit model of nucleotide substitution was HKY+I+G. Bootstrap and posterior probabilities (from Bayesian analysis) values are represented for each node.

We detected only a 3.8% prevalence of herpesvirus. A previous serologic study found no evidence of antibody to infectious laryngotracheitis virus in Imperial Cormorants and Magellanic Cormorants (Gallo et al. 2013). The cormorant herpesviruses we described belong to the subfamily *Alphaherpesvirinae* and genus *Iltovirus*, which clustered in a clade with a common ancestor with Gallid herpesvirus 1. Viruses from this family are known to cause infectious laryngotracheitis, a highly contagious herpesvirus of poultry in Chile (Servicio Agrícola y Ganadero 2010). The genetic identity among sequences of both clades was high (98%), suggesting potential transmission between both species. Because most herpesviruses are highly adapted to their host with a relatively narrow host range, we expected a higher genetic differentiation among clades from different host species. Exceptionally, cross-species transmission can occur (Kálmán and Egyed 2005; Costa et al. 2011). Further studies are required to better explore the genomic composition of these herpesviruses to understand the role of cormorants in the dissemination of poultry herpesviruses.

Herein, we have provided novel information about the diversity of viruses circulating among apparently healthy Neotropic Cormorants and contributed with molecular information that can be used for further epidemiologic and evolutionary studies. Surveillance of wild bird populations and further genomic analyses are required to understand the relationship between the viruses found in cormorants and the local virus strains of poultry, and the potential role of wild birds as reservoirs for pathogens causing emerging infectious diseases.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2017-10-256.

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