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Avian influenza virus isolated in wild waterfowl in Argentina: Evidence of a potentially unique phylogenetic lineage in South America

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ABSTRACT

Avian influenza (AI) viruses have been sporadically isolated in South America. The most recent reports are from an outbreak in commercial poultry in Chile in 2002 and its putative ancestor from a wild bird in Bolivia in 2001. Extensive surveillance in wild birds was carried out in Argentina during 2006–2007. Using RRT-PCR, 12 AI positive detections were made from cloacal swabs. One of those positive samples yielded an AI virus isolated from a wild kelp gull (*Larus dominicanus*) captured in the South Atlantic coastline of Argentina. Further characterization by nucleotide sequencing reveals that it belongs to the H13N9 subtype. Phylogenetic analysis of the 8 viral genes suggests that the 6 internal genes are related to the isolates from Chile and Bolivia. The analysis also indicates that a cluster of phylogenetically related AI viruses from South America may have evolved independently, with minimal gene exchange, from influenza viruses in other latitudes. The data produced from our investigations are valuable contributions to the study of AI viruses in South America.

Introduction

Natural infections with influenza A viruses occur in a variety of avian and mammalian hosts. Influenza A viruses of avian origin have been implicated in outbreaks of influenza in mammals, such as seals, whales, pigs, horses, mustelids, felines and humans as well as in domestic poultry species (Gauthier-Clerc et al., 2007; Ito et al., 1995; Webster et al., 1992). Influenza A viruses are classified on the basis of two glycoproteins expressed on the virus surface, the haemagglutinin (HA) and the neuraminidase (NA). To date, 16 HA (H1–H16) and 9 NA (N1–N9) antigenic subtypes have been detected in avian species and can be found in multiple combinations (Capua and Alexander, 2006; Olsen et al., 2006). In wild aquatic birds, most avian influenza (AI) infections are either subclinical or accompanied by mild clinical signs, usually caused by low pathogenicity AI strains (LPAI). Occasionally, highly pathogenic avian influenza strains (HPAI) emerge in nature and cause severe outbreaks with high mortality rates (Donis et al., 1989; Gauthier-Clerc et al., 2007).

The low virulence of AI subtypes in wild aquatic birds is thought to be the result of co-adaptation and evolution, which has allowed these viruses to find an optimal balance between high levels of replication and few pathological effects for the host (Gauthier-Clerc et al., 2007; Sharp et al., 1997; Widjaja et al., 2004). LPAI viruses have been isolated from at

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least 105 different wild bird species representing 26 families. It is widely accepted that birds of wetlands and aquatic environments such as the Anseriformes and Charadriiformes constitute the major natural reservoirs of influenza A viruses (Alexander, 2000; Olsen et al., 2006; Rappole and Hubalek, 2006). A great deal of information exists on the ecology of LPAI viruses in these reservoirs from North America and Eurasia. Surveillance studies of wild ducks in the Northern Hemisphere have revealed a seasonal infection pattern of influenza A viruses (Olsen et al., 2006). In contrast, little information is available about the presence, movement, ecology and phylogenetic relationships of LPAI viruses in wild birds in the Southern Hemisphere, particularly in South America (Donis et al., 1989; Krauss et al., 2004; Olsen et al., 2006). Waterfowl are possible reservoirs of influenza virus in South America, since there are in this region the Anatidae counterparts of the Northern Hemisphere species (De La Peña and Rumboll, 2001; Narosky, 2003). Early surveillance efforts focusing on marine birds of South America revealed negative serologic results for influenza A virus though no viral isolation was attempted (Karesh et al., 1999; Padilla et al., 2003; Travis et al., 2006; Uhart et al., 2003). Interestingly, besides historical references of HPAI outbreaks in the early 20th century the only other HPAI virus outbreak in South America was reported in 2002 from commercial poultry in Chile. Additionally, a LPAI virus H7 strain was isolated in Bolivia from an indigenous wild duck (Cinnamon Teal — *Anas cyanoptera*), which was phylogenetically related to the Chilean H7 HPAI strains (Spackman et al., 2006, 2007; Suarez et al., 2004).

Susceptibility of South American waterfowl to the Asian HPAI H5N1 strains was confirmed during a HPAI outbreak in two nature parks in Hong Kong in late 2002, when 11 Anseriformes species, indigenous to South America, were infected and died (Ellis et al., 2004). The unprecedented emergence and spread of the HPAI H5N1 virus during 2003–2004 in East and Southeast Asia and its subsequent expansion to the Middle East, Europe and Africa has placed AI on top of the priority list of potential transboundary diseases (Alexander, 2000). Whether the broad scale spread of HPAI H5N1 is due to Anatidae migrations (Gilbert et al., 2006) or human activity involved in movement of infected domestic poultry, there is certainly an urgent need to establish worldwide epidemiological efforts to track the movement and ecology of this and many other AI subtypes (Gauthier-Clerc et al., 2007). International organizations involved in human and animal health have promoted collaboration and coordinated efforts to intensify AI surveillance programs across the globe. In Argentina, the National Agriculture Technology Institute (INTA) and the National Animal Health Service (SENASA), in collaboration with the Wildlife Conservation Society (WCS) have co-organized a long-term surveillance program in resident and migratory wetland bird populations. These surveillance efforts are aimed at elucidating the presence and ecology of influenza A viruses in wild aquatic birds and to provide an early warning system for the potential introduction of HPAI strains in densely populated poultry areas (DPPAs) in Argentina. The present study reveals preliminary results obtained from the initial surveillance activities performed during 2006–2007 on hunter-killed waterfowl and captured live birds in Argentina. Using RRT-PCR, 12 AI positive samples were detected from 2895 samples. After blind passages in 10-day-old SPF chicken eggs one positive remained. The virus identified in this report, A/Kelp Gull/Argentina/LDC4/06 (H13N9), represents the first AI isolate from indigenous wild birds captured in Argentina. Sequence and phylogenetic analysis revealed similarities with other AI viruses isolated in South America, and suggest the existence of a population of AI viruses in South America that evolved independently from viruses in other latitudes.

Materials and methods

Geographic locations of sampling sites in Argentina

The targeted geographic locations included in this study are considered potential risk areas for avian influenza infections and outbreaks (Fig. 1). The sampling locations consist of vast wetlands located along important migratory bird pathways and in close proximity to Densely Populated Poultry Areas (DPPAs), which comprise approximately 70% of the poultry production in Argentina. These wetland locations serve as reservoirs for multiple populations of resident waterfowl species, particularly ducks (*Netta peposaca*, *Amazonetta brasiliensis*, *Anas versicolor*, etc.). From October to March (Austral spring and summer seasons), a large number of gregarious migratory Charadriiformes use these wetlands as feeding grounds or stopover sites, interacting with resident waterfowl populations. In addition, agro-ecosystems, including rice plantations, receive significant numbers of Nearctic waders during December and January. These habitats are extensive along the Argentine Atlantic coast and in addition, vast fresh water wetlands, consisting of lagoons, forest islands and marshes are interconnected via the Paraná and Uruguay rivers, two of the major river systems in South America. Contrary to the Northern Hemisphere, South American duck populations have a more homogeneous distribution of the number of birds during the year, as winter migrations are not as critical (López-Lanús and Blanco, 2005; Olsen et al., 2006; Gilbert et al., 2006). From May to August, the wetlands are used for game bird hunting focusing mainly on ducks, tinamous and wild pigeons.

Two major geographic areas were used as sampling sites (Fig. 1).

Area 1 includes four districts of Entre Ríos province (Gualeguay, Paraná, La Paz and Victoria), one district in Corrientes province (Esquina) and one district in Santa Fe province (San Javier). Samples were collected from private hunting lodges and national reserves, strategically set within the described wetlands. Each of these 6 districts covers 15,000 hectares. In area 2 samples were obtained from a variety of seabirds (gulls, penguins, cormorants, terns and shorebirds) captured along the Argentine Atlantic coast from the provinces of Buenos Aires to Tierra del Fuego. This particular seashore area covers approximately 5000 km from parallel 36°S to 55°S.

Sample collection

Trained biologists and veterinarians carried out the sampling activities. Each bird was initially identified by species, sex, age category (adult, juvenile or fledgling) and weight (Tables 1A and 1B). Following identification swabs were collected. Cloacal swabs, 2895 in total, were collected from wild waterfowl using single-use polyester sterile swabs and then stored separately into single plastic cryo-tubes, containing 2 ml of Phosphate Buffer Solution (PBS) with 50% glycerol and Penicillin 10,000 IU/ml, Streptomycin 5000 μg/ml, Gentamicin Sulfate 1000 μg/ml, Kanamycin sulfate 700 μg/ml and Anphotericin B 10 μg/ml (Sigma Chemical Co™, St. Louis, MO, USA). Samples were either refrigerated at 4 °C for less than 48 h before they were processed for molecular diagnosis and virus isolation or, if immediate processing was not available, they were frozen in liquid nitrogen.

AI detection

Viral RNA was extracted from 200 μl of PBS suspension from cloacal swabs using the Total RNA Isolation Chemistry Starter Kit (Applied Biosystems™, Foster City, CA, USA) in accordance with the manufacturer's instructions. RNA was eluted in a final volume of 100 μl and stored at −80 °C. Viral cDNA was prepared using 30 μl of viral RNA and random hexamers in a final volume of 60 μl as per manufacturer's directions using the High Capacity cDNA Archive kit™ (Applied Biosystems™, Foster City, CA, USA). The cDNA was tested for AI by real-time reverse transcription PCR (RRT-PCR) using TaqMan Universal PCR Master Mix™ (Applied Biosystems™, Foster City, CA, USA) directed to the matrix (M) gene, which detects all type A influenza viruses as previously reported (Spackman et al., 2002). The PCR reaction was performed in an ABI Prism 7500 SDS apparatus (Applied Biosystem™, Foster City, CA, USA).

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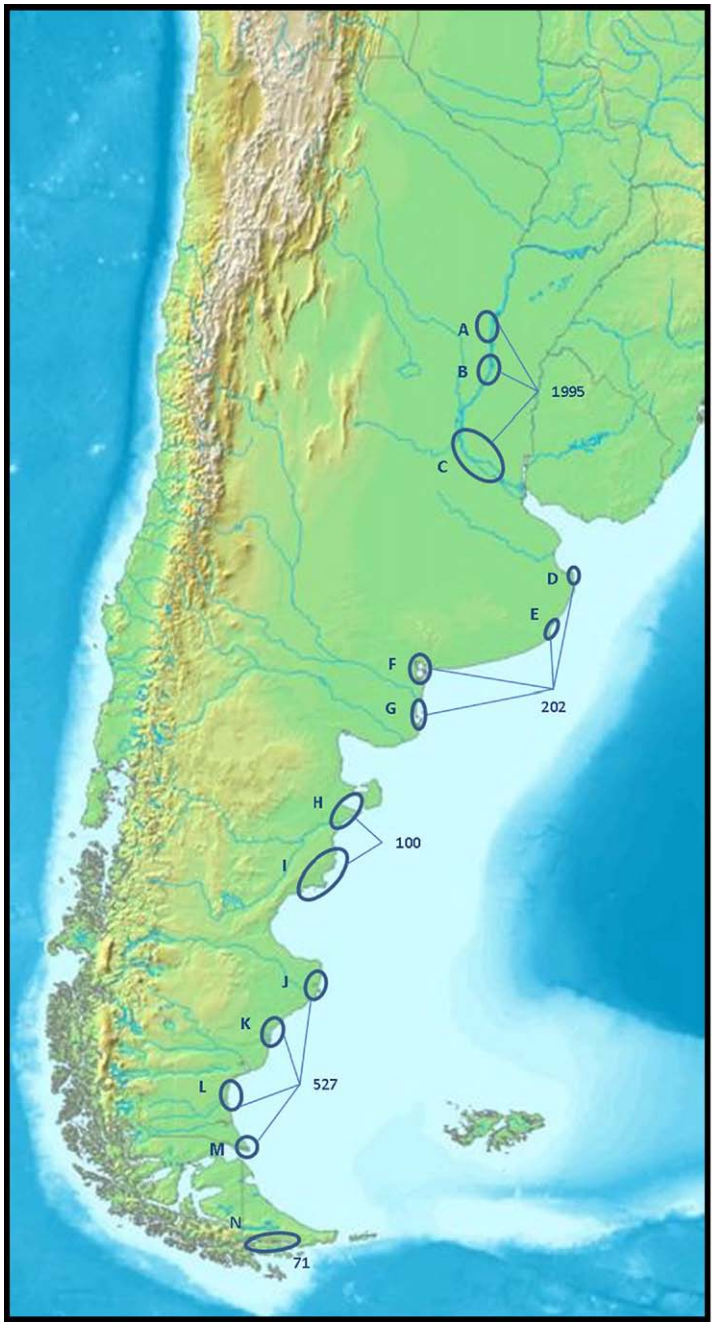

#	Location	Province	Positive RRT-PCR
A	Esquina	Corrientes	3
B	San Javier La Paz	Santa Fe Entre Ríos	5 2
C	Victoria	Entre Ríos	1
D	Pta. Rasa	Buenos Aires	0
E	Mar Chiquita	Buenos Aires	0
F	Bahía Blanca	Buenos Aires	1
G	Bahía San Blas	Buenos Aires	0
H	Pta Loma – Pta. León	Chubut	0
I	Pta Tombo - Cb. Dos Bahías - Bahía Bustamante	Chubut	0
J	Puerto deseado	Santa Cruz	0
K	Bahía San Julián	Santa Cruz	0
L	Monte León	Santa Cruz	0
M	Cabo Vírgenes	Santa Cruz	0
N	I. Conejo, I. Martillo, La Turbera	Tierra del Fuego	0

Fig. 1. Geographical location of the areas under surveillance. The numbers correspond to the quantity of samples analyzed from each area. The associated table indicates the locations individualized from A to N, with the positive RRT-PCR results obtained from each of them.

Virus isolation and characterization

All swab samples determined positive by RRT-PCR were processed for virus isolation in specific pathogen free (SPF) embryonated chicken eggs. Briefly, 200 µl of PBS suspension from the cloacal swab samples were used for viral isolation in the allantoic cavity of 9–11 day-old SPF chicken embryonated eggs, in accordance to standardized protocols described in the OIE (2000) and to Argentine regulations.

Genetic analysis and phylogenetic characterization

The genome segments of AI were amplified by RT-PCR as described by Hoffmann et al. (2001) and directly sequenced with the BigDye terminator kit (Applied Biosystems™, Foster City, CA, USA) on an ABI 3730 (Applied Biosystems™, Foster City, CA, USA). Regions corresponding to the entire open reading frame of HA, NP, NA, M1 and NS, and partial sequences of PB2, PB1 and PA were obtained (primer sequences available upon request). Genomic information was derived from overlapping sequences covered by forward and reverse primers. At least two independent RT-PCR reactions were produced for each gene and used for sequencing. Sequences are available through GenBank, accession nos. EU523136 through EU523143. Nucleotide BLAST analysis (at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was initially used to identify the most closely related influenza A virus gene for each respective segment. Then, all available isolates obtained were included in a multiple alignment by CLUSTAL X Version 1.8.3 program (Thompson et al., 1994), and the percent identities were calculated. The same reference isolates were then used for phylogenetic analysis. The phylogenetic trees, calculated by neighbor-joining method, were computed with the DNADIST and NEIGHBOR modules

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Table 1A
Details of birds sampled in the present study, indicating the scientific and common name, the order and family and the number of birds sampled

Scientific name	Common name	Samples[a]	Order	Family
Amazonetta brasiliensis	Brazilian duck	143	Anseriformes	Anatidae
Anas bahamensis	White-cheeked pintail	5	Anseriformes	Anatidae
Anas flavirostris	Speckled teal	16	Anseriformes	Anatidae
Anas platalea	Red shoveler	9	Anseriformes	Anatidae
Anas sibilatrix	Southern wigeon	1	Anseriformes	Anatidae
Anas versicolor	Silver teal	140	Anseriformes	Anatidae
Callonetta leucophrys	Ringed teal	103	Anseriformes	Anatidae
Coscoroba coscoroba	Coscoroba swan	1	Anseriformes	Anatidae
Dendrocygna bicolor	Fulvous whistling duck	139	Anseriformes	Anatidae
Dendrocygna viduata	White-faced whistling duck	53	Anseriformes	Anatidae
Heteronetta atricapilla	Black-headed duck	1	Anseriformes	Anatidae
Netta peposaca	Rosy-billed pochard	1242	Anseriformes	Anatidae
Oxyura dominica	Masked duck	1	Anseriformes	Anatidae
Sarkidiornis melanotos	Comb duck	1	Anseriformes	Anatidae
Anas georgica	Brown pintail	4	Anseriformes	Anatidae
Dendrocygna autumnalis	Black-bellied whistling duck	1	Anseriformes,	Anatidae
Calidris fuscicollis	White-rumped sandpiper	9	Charadriiformes	Scolopacidae
Calidris melanotos	Pectoral sandpiper	16	Charadriiformes	Scolopacidae
Charadrius collaris	Collared plover	5	Charadriiformes	Charadriidae
Larus atlanticus	Olrog's gull	73	Charadriiformes	Laridae
Larus dominicanus	Kelp gull	162	Charadriiformes	Laridae
Larus scoresbii	Dolphin gull	15	Charadriiformes	Laridae
Nycticryphes semicollaris	South American painted snipe	2	Charadriiformes	Rostratulidae
Pluvialis dominica	American golden plover	3	Charadriiformes	Charadriidae
Rynchops niger	Black skimmer	6	Charadriiformes	Rhynchopidae
Thalasseus s. eurygnatha	Cayenne tern	17	Charadriiformes	Sternidae
Sterna hirundinacea	South American tern	91	Charadriiformes	Sternidae
Thalasseus maximus	Royal tern	36	Charadriiformes	Sternidae
Thalasseus sandvicensis	Sandwich tern	42	Charadriiformes	Sternidae
Tryngites subruficollis	Buff-breasted sandpiper	8	Charadriiformes	Scolopacidae
Ardea cocoi	White-necked heron	1	Ciconiiformes	Ardeidae
Ardea alba	Great egret or white heron	1	Ciconiiformes	Ardeidae
Phimosus infuscatus	Bare-faced ibis	1	Ciconiiformes	Threskiornithidae
Plegadis chihi	White-faced ibis	5	Ciconiiformes	Threskiornithidae
Columba maculosa	Spotted-winged pigeon	3	Columbiformes	Columbidae
Columbina picui	Picui ground-dove	11	Columbiformes	Columbidae
Fulica leucoptera	White-winged coot	16	Gruiformes	Rallidae
Fulica rufifrons	Red-fronted coot	1	Gruiformes	Rallidae
Furnarius rufus	Rufous hornero	3	Passeriformes	Furnariidae
Mimus saturninus	Chalk-browed mockingbird	2	Passeriformes	Mimidae
Molothrus bonariensis	Shiny Cowbird	2	Passeriformes	Icteridae
Polioptila dumicola	Masked gnatcatcher	2	Passeriformes	Sylviidae
Progne modesta	Southern martin	19	Passeriformes	Hirundinidae
Saltator coerulescens	Greyish saltator	1	Passeriformes	Thraupidae
Synallaxis frontalis	Sooty-fronted spinetail	2	Passeriformes	Furnariidae
Taraba major	Great antshrike	1	Passeriformes	Thamnophilidae
Turdus amaurochalinus	Creamy-bellied thrush	10	Passeriformes	Turdidae
Turdus rufiventris	Rufous-bellied thrush	2	Passeriformes	Turdidae
Zonotrichia capensis	Rufous-collared sparrow	1	Passeriformes	Emberizidae
Phalacrocorax albiventer	King cormorant	17	Pelecaniformes	Phalacrocoracidae
Phalacrocorax atriceps	Imperial cormorant	78	Pelecaniformes	Phalacrocoracidae
Phalacrocorax magellanicus	Rock cormorant	1	Pelecaniformes	Phalacrocoracidae
Phalacrocorax brasilianus	Neotropic cormorant	1	Pelecaniformes	Phalacrocoracidae
Macronectes giganteus	Southern giant petrel	18	Procellariiformes	Procellariidae
Spheniscus magellanicus	Magellanic penguin	349	Sphenisciformes	Spheniscidae
Nothura maculosa	Spotted tinamou	2	Tinamiformes	Tinamidae

[a] Number of samples obtained.

of the PHYLIP package (Felsenstein, 1989). Bootstrapping values (1000 replicates) were calculated with the modules SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. Branches with bootstrapping values ≥70 were considered significant, corresponding to a confidence interval ≥95% (Hillis and Bull, 1993). For visualization and printing of the trees, the TREEVIEW program, Version 1.6.6 (Page, 1996) was used.

Results

Isolation of a H13N9 type A influenza virus from a Kelp Gull

From March 2006 to December 2007, 2895 cloacal swabs were collected from wild waterfowl from different locations (as described previously in Materials and methods). Our sampling and processing

Table 1B
Table summarizing the number of samples for each order and the percentage from the total of samples obtained

Order	Samples[a]	Percentage
Anseriformes	1860	64,2
Charadriiformes	485	16,8
Ciconiiformes	8	0,3
Columbiformes	14	0,5
Gruiformes	17	0,6
Passeriformes	45	1,6
Pelecaniformes	97	3,4
Procellariiformes	18	0,6
Sphenisciformes	349	12,1
Tinamiformes	2	0,1
TOTAL	2895	100.0

[a] Number of samples obtained.

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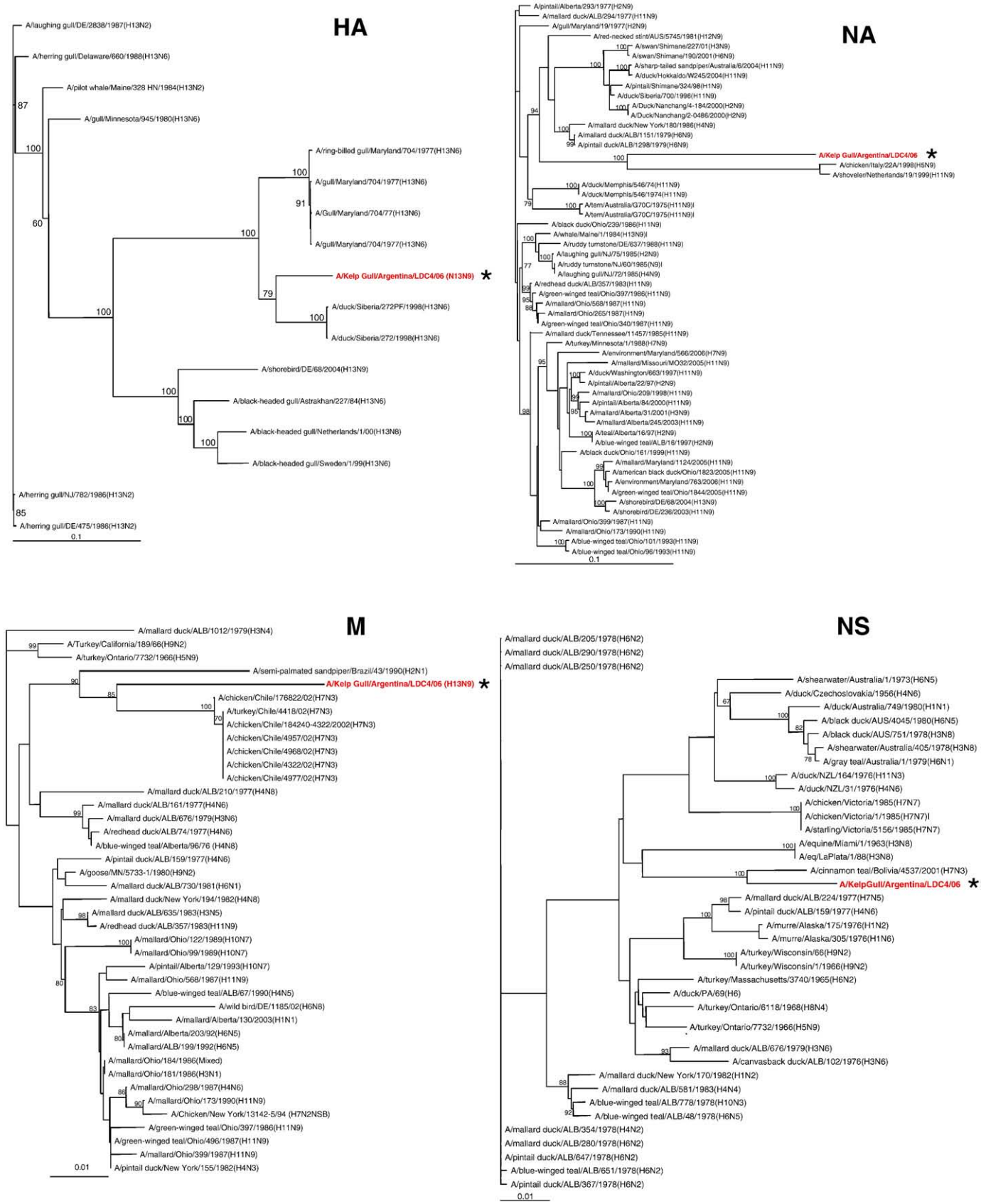

Fig. 2. Phylogenetic trees of individual gene segments (indicated in the upper-right side of each tree). All trees were constructed with PHYLIP package (Felsenstein, 1989) using Neighbor Joining with 1000 bootstrap replicates. Bootstrap values above 60% are detailed. To easily identify the Argentinean isolate an asterisk (*) was used.

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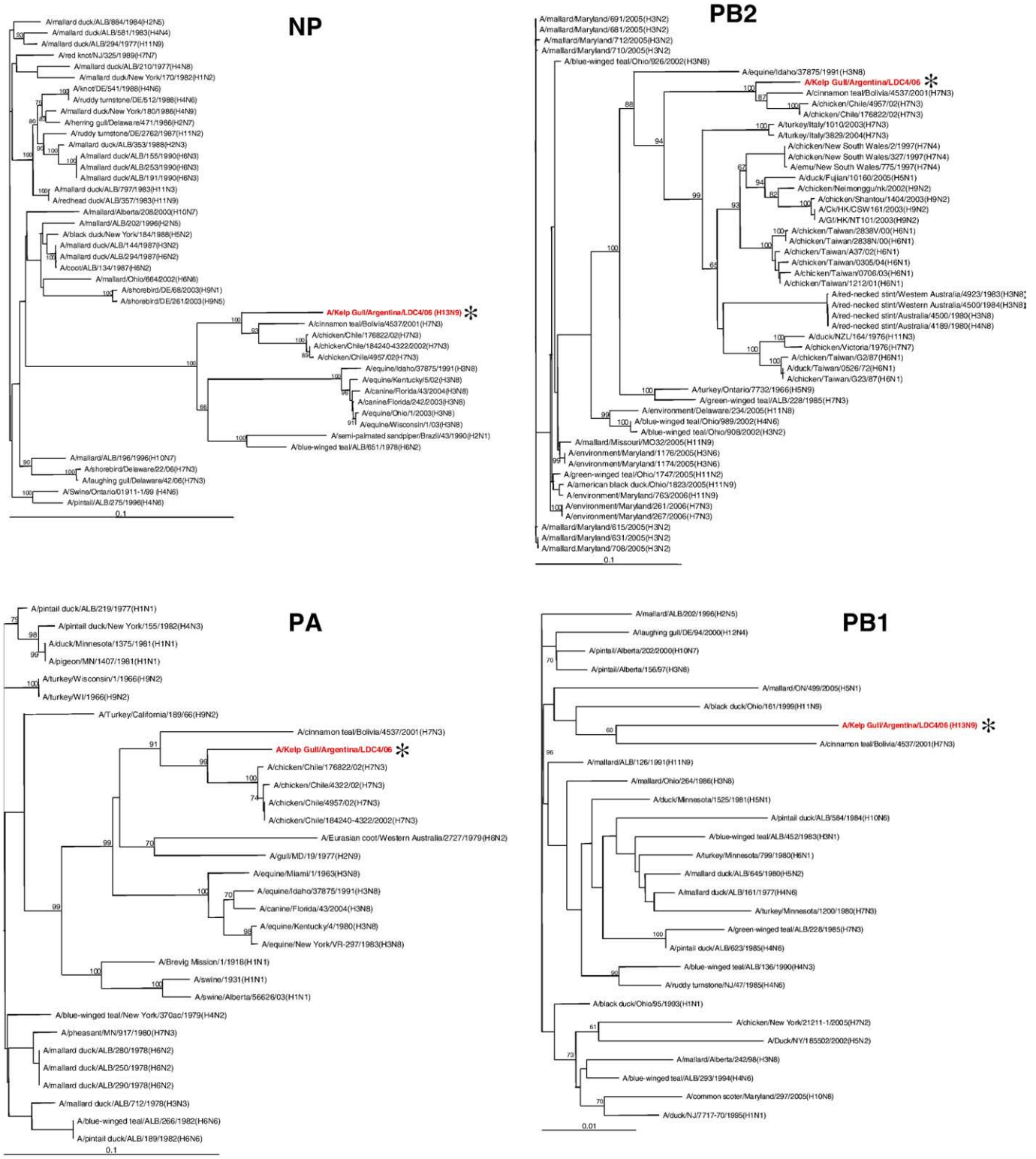

Fig. 2 (*continued*).

procedures revealed the presence of 12 AI positive samples by RRT-PCR, using the matrix gene as the target for amplification. Additional RRT-PCR tests ruled out the presence of H5 and H7 using a protocol kindly provided by Dr. William Dundon at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVE, 2006). Eleven of the twelve RRT-PCR positives samples corresponded to Area 1 and one to Area 2 (Fig. 1). Efforts to grow the RRT-PCR positive samples in SPF embryonated chicken eggs resulted in only one positive isolation from Area 2. This virus was isolated from a free-ranging Kelp Gull (*Larus dominicanus*) captured in the estuary of Bahía Blanca, in the southern part of Buenos Aires province (40° 33′S, 62° 16′W). Initial molecular characterization of this isolate revealed that it is an influenza type A virus of the H13N9 subtype and was named A/Kelp Gull/Argentina/LDC4/06 (H13N9). The Kelp Gull is mainly a coastal gull, which breeds on shores and islands throughout much of the southern hemisphere. *L. dominicanus* is a subspecies found mostly in

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South America, parts of Australia and New Zealand (where it is also known as the Southern Black-backed Gull or Kororo). It is the southern equivalent of the northern hemisphere's Lesser Black-backed Gull and is similar in size and wingspan. Our study suggests that the Kelp Gull of the Argentine Atlantic coast is a potential reservoir of avian influenza viruses in South America. To our knowledge this is the first avian influenza virus isolated from gulls in South America.

The detection rate using RRT-PCR was 0.41%, whereas isolation in SPF eggs yielded an overall virus prevalence of 0.035%. This and previous studies suggest that it is not possible to rely singly on RRT-PCR or virus isolation to perform thorough characterizations of avian influenza viruses in wild birds (Runstadler et al. 2007). The H13N9 virus isolated in this study was obtained after a second blind passage in chicken embryos producing HA titers between 1/64 and 1/128 and a virus titer of 10^5 EID50/ml (mean egg infectious dose). Thus, it is tempting to speculate that failure to amplify some of our RRT-PCR positives in SPF chicken eggs maybe due to the inability of the latter to act as a good substrate for virus growth.

Sequencing and phylogenetic characterization of the H13N9 from Kelp Gull suggest the presence of influenza genes unique to the Southern Hemisphere

In order to better characterize the H13N9 virus isolated from Kelp Gull, we performed sequencing and phylogenetic analysis for the 8 genomic segments. Based on the phylogenetic analysis performed in this study (Fig. 2), the PB2, PB1, PA, NP, M1 and NS genes are most closely related, phylogenetically, to the AI isolates collected during an outbreak in commercial poultry in Chile in 2002 and the putative ancestor isolated from a Cinnamon Teal in Bolivia (Table 2). In addition, the NP and M genes are more distantly related to an AI virus isolated from a shorebird in Brazil in 1990, A/semi-palmated sandpiper/Brazil/43/1990 (H2N1) (Fig. 2). The analysis also indicates that the internal genes from the South American isolates are grouped in their own cluster with considerable bootstrap values (Fig. 2). The PA, NP and NS genes also showed phylogenetic relationship with influenza viruses isolated from equine and canine species; most notably the NS tree shows a close relationship with an Argentinean equine isolate. Spackman et al. (2006) previously described a similar relationship in the NS gene for the Bolivian isolate. This data suggests the potential for occasional introduction of AI genes from birds into the horse population in South America.

With respect to genes that code for surface proteins, the closest HA gene relative is A/duck/Siberia/272PF/1998 (H13N6) while bootstrap values indicate a more distant relationship to H13 viruses isolated from gulls in Maryland, USA in 1977. Interestingly, our kelp gull isolate is less phylogenetically related to more recent H13 viruses isolated in North America or Europe. Thus, it is tempting to speculate that H13 viruses in South America may have evolved independently from other H13 viruses in other parts of the world. Analysis of the deduced HA protein sequence shows a cleavage site consistent with a low pathogenic AI (VPAISNRGLF) with little variation among viruses of the H13 subtype (Table 3). Interestingly, the N9 NA gene is more closely related to European isolates than to North American isolates.

Table 2
Type A Influenza virus isolates most related to A/KelpGull/Argentina/LDC4/06 by individual gene segment

Gene segment	Most related virus isolate	Percentage of identity (percentage sequenced)
4 — HA	A/duck/Siberia/272/1998 (H13N6)	91 (100)
6 — NA	A/shoveler/Netherlands/19/1999(H11N9)	78 (94)
8 — NS	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	97 (100)
7 — M	A/chicken/Chile/184240-4322/2002(H7N3)	96 (50)
3 — PA	A/chicken/Chile/184240-4322/2002(H7N3)	95 (26)
2 — PB1	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	94 (28)
1 — PB2	A/chicken/Chile/176822/02(H7N3)	94 (29)
5 — NP	A/cinnamon teal/Bolivia/4537/2001 (H7N3)	94 (100)

Table 3
Amino acidic sequence pattern of the cleavage site of all available H13 HA subtype Influenza viruses

A/duck/Siberia/272/1998(H13N6)	VPAISNRGLF
A/gull/Maryland/704/1977(H13N6)	VPAISNRGLF
A/black-headed gull/Netherlands/1/00(H13N8)	VPAISKRGLF
A/black-headed gull/Sweden/1/99(H13N6)	VPAISNRGLF
A/herring gull/DE/475/1986(H13N2)	VPATSNRGLF
A/shorebird/DE/68/2004(H13N9)	VPAIASRGLF
A/herring gull/NJ/782/1986(H13N2)	VPATSNRGLF
A/laughing gull/DE/2838/1987(H13N2)	VPSTSNRGLF
A/herring gull/Delaware/660/1988(H13N6)	VPATSNRGLF
A/gull/Maryland/704/1977(H13N6)	VPAISNRGFF
A/gull/Minnesota/945/1980(H13N6)	VPATSNRGLF
A/Gull/Maryland/704/77(H13N6)	VPAISNRGLF
A/ring-billed gull/Maryland/704/1977(H13N6)	VPAISNRGLF
A/pilot whale/Maine/328 HN/1984(H13N2)	VPAISNRGLF
A/Kelp Gull/Argentina/LDC4/06 (H13N9)	**VPAISNRGLF**

The two most phylogenetically related NA genes are from influenza viruses isolated during a poultry outbreak in Italy and from a wild duck captured in the Netherlands (Fig. 2). The NA phylogenetic tree shows that these isolates are grouped in a different cluster from others N9 subtype viruses and supported with high bootstrap values, indicating a phylogenetically distinct lineage group of N9 NA genes. However, the nucleotide distance between the European and the Argentinean isolates is remarkably high (22%) to support a unique lineage group and additional N9 sequences from this cluster would be necessary to better explain its evolutionary pattern.

Discussion

As noted previously (Spackman et al., 2006, 2007), AI surveillance in wild birds in South America has been minimal. In this study we attempted to provide a better understanding of the ecology of AI in wild birds in Argentina. National Veterinary Services in South American countries are routinely screening commercial and backyard poultry farms for the presence of AI. These efforts are targeted to prevent the introduction of AI in domestic poultry. However, in order to provide a better and more credible risk analysis of the potential introduction of AI to commercial and/or backyard poultry it is necessary to monitor the presence and prevalence of these viruses in wild bird populations (Munster et al., 2006). One H7N3 HPAI outbreak in commercial poultry was reported in Chile in 2002 (Suarez et al., 2004). The source of the virus was postulated to be wild waterfowl due to the low infectivity of this virus strain in chickens. Interestingly a closely related LPAI H7 virus was obtained from a Cinnamon Teal in Bolivia collected only a few months before the HPAI outbreak. However, this virus was only characterized recently (Spackman et al., 2006, 2007) highlighting the importance of timely wild bird surveillance and virus characterization.

The present study reveals data obtained from the initial, inter-institutional AI surveillance effort performed in Argentina during 2006–2007 on hunter-killed waterfowl and captured live birds. As a result, we report for the first time an isolation of a H13N9 AI virus from a kelp gull in Argentina. As past surveillance efforts have shown, a majority of the H13 viruses isolated to date have been from different gull species and this case is not an exception (Fouchier et al., 2005). However, our phylogenetic analysis suggests that the H13 virus isolated in Argentina is evolving independently from other H13 viruses. More importantly, our analysis indicates that the Kelp Gull N9 gene belongs to a phylogenetic cluster that until recently contained NAs of purely Eurasian origin, leading us to believe that this lineage spreads beyond Eurasia. However, additional N9 gene sequences from this poorly defined lineage are required to draw proper conclusions based on the high nucleotide distance observed between the European and Argentinean isolates. It is also interesting to note that

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the internal genome segments (PB2, PB1, PA, NP, M, and NS) of this isolate are closely related to those viruses previously isolated from the Chilean outbreak, and the putative ancestor from a wild bird captured in Bolivia. This relation suggests a pattern of genetic evolution with little exchange of segments with North American isolates, despite sharing multiple trans-hemispheric migratory routes. As noted by others (Spackman et al., 2006) the NS genes from these South American isolates are related to equine viruses; however it is not a gene derived from the equine lineage due the great nucleotide divergence observed. Our data coupled with the fact that the kelp gull (*L. dominicanus*) is a nonmigratory bird, supports the notion of a particular, and yet poorly understood, ecology of AI viruses in South America. In this regard, this study certainly sheds more attention and awareness regarding the presence and molecular characteristics of AI viruses in South America.

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