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First isolation of an H1N1 avian influenza virus from wild terrestrial non-migratory birds in Argentina

Paula Alvarez [a], Rosana Mattiello [b], Pierre Rivailler [c], Ariel Pereda [d], Charles T. Davis [c], Lorena Boado [a], Elisa D'Ambrosio [b], Sebastian Aguirre [a], Cora Espinosa [e], José La Torre [a], Ruben Donis [c], Nora Mattion [a,*]

[a] Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. César Milstein, CONICET, Saladillo 2468, (1440) Buenos Aires, Argentina
[b] Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Chorroarín 280, (1427) CABA, Argentina
[c] Influenza Division, Centers for Disease Control and Prevention, 1600 Clifton Road, NE, Atlanta, GA 30333, USA
[d] Instituto Nacional de Tecnología Agropecuaria, Centro de Investigación de Ciencias Veterinarias y Agronómicas (INTA-CICVyA), CC77, (1708) Morón, Argentina
[e] Servicio Nacional de Sanidad Animal (SENASA), Av. Paseo Colon 367, (1063) Buenos Aires, Argentina

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ABSTRACT

A type A avian influenza (AI) virus was isolated from dead or severely ill red-winged tinamous (*Rhynchotus rufescens*) found in a hunting ground in April 2008 in Argentina. The subtype of A/red-winged tinamou/Argentina/MP1/2008 was determined as H1N1 by sequence analysis. The cleavage site of the viral hemagglutinin corresponded to a low pathogenic influenza virus, although the clinical presentation and pathological studies suggest that the virus was pathogenic for red-winged tinamous. Phylogenetic analysis of the viral genome suggested that while the hemagglutinin and neuraminidase genes were related to AIV from North America, the internal genes were most closely related to other South American isolates. These findings support the postulated South American phylogenetic lineage for AIV PB2, PB1, PA, M and NS genes, and suggest that the evolutionary pathways of HA and NA genes involve exchanges between the Northern and Southern hemispheres.

Introduction

Wild waterfowl carry and transmit a wide range of influenza A viruses in nature (Slemons et al., 1974, Webster et al., 1992, Webster and Hulse, 2004). Indeed, all 16 HA and 9 NA subtypes of type A viruses are maintained in aquatic bird populations, which may excrete large amounts of virus for long periods via feces or respiratory secretions (Poland et al., 2007, Runstadler et al., 2007, Webster et al., 1992). Migratory birds, notably waterfowl, are usually infected with low-pathogenic avian influenza (LPAI) viruses. Although these infections are usually subclinical (Swayne, 2008), it has been suggested that LPAI viruses in wild migratory birds may have higher clinical and ecological impact than previously recognized (van Gils et al., 2007).

Transmission of AI viruses from wild to domestic birds with subsequent mutation from LPAI to HPAI has been reported with increasing frequency (Munster et al., 2005). Since its emergence in 1997, the highly pathogenic AI virus of the subtype H5N1 has became enzootic in parts of Asia and Africa (Cauthen et al., 2000; Li et al., 2004). Although virulence is likely to be polygenic (De Wit et al., 2008), the HA protein might play a pivotal role. The cleavability of HA into HA1 and HA2 subunits is enhanced by the insertion of multiple basic amino acids (lysine and arginine) at the cleavage site. Circulation of AIV in terrestrial birds is thought to play an essential role in the acquisition of multibasic cleavage sites in the HA of subtype H5 and H7 viruses, and the HP phenotype (Banks et al., 2001, Perdue et al., 1996, Suarez et al., 2004). Equivalent conversion of LP AIV to the HP phenotype has not been reported for other subtypes; e.g. H4 and H6, despite circulation in domestic terrestrial birds (Donis et al., 1989; Webby et al., 2002). However, as was reported in Southern China, continuous cocirculation of H5N1, H6N1/N2 and H9N2 influenza viruses led to frequent reassortment in minor poultry species that greatly increased the genetic diversity and complexity of influenza virus in this region, thereby increasing the chance of emergence of influenza viruses with pandemic potential (Cheung et al., 2007; Xu et al., 2007). Interestingly, wild terrestrial birds have not been implicated in the emergence of HPAI viruses.

Although Newcastle Disease virus (NDV) was reported in South America more than three decades ago, the first report of avian influenza virus in commercial chickens was in 2002 (Suarez et al., 2004). LPAIV subtype H7N3 was isolated from a broiler breeder flock in Chile [A/chicken/Chile/176822/02 (H7N3)], and 1 month later, an HPAI virus of the same subtype was isolated in the same premises [A/chicken/Chile/4957/2002(H7N3)] (Rojas et al., 2002). Analysis of the HA gene indicated that Chilean H7N3 HPAI viruses have emerged from the LPAI viruses (Suarez et al., 2004). In further studies, these isolates were found to be phylogenetically related to the earliest AIV isolated from a

* Corresponding author. Fax: +54 11 4686 6225.
E-mail address: nmattioncevan@centromilstein.org.ar (N. Mattion).

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wild duck, cinnamon teal (*Anas cyanoptera*) in South America [A/cinnamon teal/Bolivia/4537/2001 (H7N3)] (Spackman et al., 2006).

In Argentina, a surveillance program for AI in backyard poultry was conducted between 1998 and 2005 (Buscaglia et al., 2007). Evidence of serum antibodies to AIV was not found in 8000 birds tested by ELISA and/or agar gel immunodiffusion. Likewise, no AIV was isolated from 18,000 tracheal and cloacal swabs (Buscaglia et al., 2007). More recently, in the years 2006 and 2007, a virologic survey of 2995 waterfowl and shorebirds harvested by hunting or live-captured, revealed 12 samples positive for influenza A virus by RRT-PCR (Pereda et al., 2008). These specimens yielded only one virus isolation, from a free ranging kelp gull (*Larus dominicanus*) captured in a southern district of Buenos Aires Province [A/kelp gull/Argentina/LDC4/2006 (H13N9)].

In April 2008, several wild red-winged tinamous (*Rhynchotus rufescens*, Tinamiformes order, Tinamidae family) were found dead in the surroundings of Marcos Paz district, located 50 km from the Rio de la Plata coastline, in the Province of Buenos Aires, with signs and lesions of acute respiratory disease compatible with viral infection. A type A AI virus was isolated from the affected birds.

Tinamous have a unique evolutionary position among the bird kingdom. Although they look similar to other ground-dwelling birds like quail and grouse, tinamids have no close living relatives, and hence were placed in their own order, Tinamiformes (Gotch 1995). A recent phylogenomic study on birds showed that the Tinamiformes order lies within the Struthioniformes order (Hackett et al., 2008). The study showed tinamids as the sister group of Australasian/Oceanian ratites (cassowaries, emus, and kiwi), with South American ratites (rheas) and African ratites (ostriches) as successive outgroups (Hackett et al., 2008). South America has two species of rhea and their closest living relatives are the tinamous (Gotch, 1995; Gauthier and de Queiroz, 2001). The family Tinamidae consists of about 47 species in 9 genera. The red-winged tinamou (*Rhynchotus rufescens*) belongs to the *Rhynchotus* genus of the Tinamidae family. They have very small wings, but unlike other ratites, they can fly, albeit poorly.

This is the first report of an AIV isolated from land-based non-migratory wild birds in South America and the first isolate from the Tinamiformes order worldwide.

Results

Investigation of the cluster of respiratory disease

Despite the presence of different species of birds in the area (see Materials and methods and Figure S2), only red-winged tinamous (*Rhynchotus rufescens*) were found clinically affected, showing acute respiratory distress syndrome. The animals showed signs of lethargy, sneezing, oculo-nasal discharge, swelling of the sinuses and difficult breathing (the head and neck were held in extension) and passage of greenish-colored feces. Some birds developed neurologic signs that included ataxia and convulsions. Most affected birds died within one or two days after developing clinical signs.

A subsequent survey was performed in June 2008 by acting veterinarians, including red-winged tinamous, six aquatic bird species (listed in Materials and methods) captured in the same hunting area and five tinamous from an irregular neighbor breeder. Additionally, an official survey was conducted by the Argentine Animal Health Service (SENASA) including backyard poultry and one large commercial laying hens establishment with negative results (data not shown).

Histopathological analysis

Gross lesions identified among severely ill tinamou included an accumulation of necrotic debris in the sinuses, air sacs and trachea; swelling, congestion and hemorrhage of the liver and lungs (Supplementary Figure 3). Other gross changes included swelling of brain, excess mucus in the small intestine, and swollen, pale kidneys with abundant whitish precipitates and splenomegaly. Microscopic observation of the lungs showed severe diffuse congestion with multifocal hemorrhages and edema. The bronchial epithelium was hyperplasic and infiltrated by lymphocytes. Some lungs showed mild to moderate interstitial pneumonia and others had necrosis of air and blood capillary endothelium. Spleens presented widespread endothelial swelling and proliferation with fibrinoid necrosis of splenic arterioles, apoptotic cells and lymphocyte necrosis with reactive histiocytic hyperplasia (Supplementary Figure 4). Histological changes of the liver were characterized by diffuse congestion, with different degrees of multifocal coagulative necrosis, kariorrhexis, kariolisis, megalocitosis and apoptosis of the hepatocytes. Fatty changes, as well as the periportal mononuclear cell infiltration and scattered heterophils were seen (Supplementary Figure 5). In the kidneys, diffuse tubular degeneration, granular and hyaline casts were found in all samples. Some of them showed tubular necrosis with megalocytosis and lymphoplasmacytic interstitial nephritis. Few samples had necrosis of the lymphoid tissue in the intestine, and crypts containing exfoliated cells. Diffuse necrosis of the pancreatic exocrine cells and peritubular mononuclear cell infiltration were seen in only in one sample (data not shown).

Molecular diagnosis by RT-PCR in organ specimens

A segment of 640 bp of the HA gene was amplified with the type A universal primer pair from RNA extracted from tinamou organ samples and cloacal or tracheal swabs (Supplementary Figure 6A, lane 1). The sequence of the PCR products identified the virus as influenza A virus of subtype H1 (data not shown).

Amplification of fragments of the expected size (616 bp) with the N1 specific primer pair was obtained from lung and liver specimens (Supplementary Figure 6B, line 1). The sequence of these PCR products confirmed the virus subtype as N1 (data not shown). The isolate was thus characterized as belonging to the subtype H1N1 and denominated A/red-winged tinamou/Argentina/MP1/2008 (H1N1).

Cloacal and tracheal swabs from all birds captured alive in June 2008 during the second survey were analyzed as described above, but influenza viruses were not detected in these samples (data not shown).

AI isolation from tissue extracts

Virus was recovered from lungs and liver extracts of dead birds (April 2008) after inoculation into 10-day-old embryonated chicken eggs. The embryos were killed by the virus at 48–72 h post-inoculation and the allantoic fluids were collected for further characterization by RT-PCR and gene sequencing.

Allantoic fluids from eggs inoculated with tissue homogenates from tinamous affected by respiratory disease in April 2008, revealed HA activity. In contrast, eggs inoculated with cloacal and tracheal swabs from live-captured red-winged tinamous sampled in June 2008 were negative (data not shown).

Table 1
Hemagglutination inhibition (HI) testing of sera from birds caught alive 2 months after AI isolation.

Bird species	HI (positives/total)
Red-winged tinamou (*Rhynchotus rufescens*)	19/24
White-faced tree-duck (*Dendrocygna viduata*)	0/5
Brown pintail (*Anas georgica*)	0/5
Speckled teal (*Anas flavirostris*)	0/5
White-winged coot (*Fulica leucoptera*)	0/5
White-cheeked pintail (*Anas bahamensis*)	0/5
Coscoroba swan (*Coscoroba coscoroba*)	0/6

Serological analysis

The presence of haemagglutination inhibition antibodies in the serum of samples collected in the hunting ground in June 2008 was analyzed. All red-winged tinamou serum samples collected from free-flying birds showed HI titers (19 birds, Table 1), demonstrating that although virus was not isolated in embryonated eggs or detected by RT-PCR at this time, several birds had been previously infected by AIV. Positive HI titers ranged from 40 to 2560. As some tinamous found in the hunting ground might be originated in occasional breeders, other five serum samples were collected, in the same district, from tinamous raised in captivity. These animals never presented signs of disease and tested negative (Table 1).

Additionally, five samples from individual birds of several free flying aquatic species (listed in Materials and methods) from the hunting ground were tested by HI, with negative results (Table 1).

All the bird serum samples resulted negative for NDV by the HI test (data not shown). Taken together, the serological results are consistent with the localized and transient circulation of this virus in tinamous in the Fall of 2008 in Argentina.

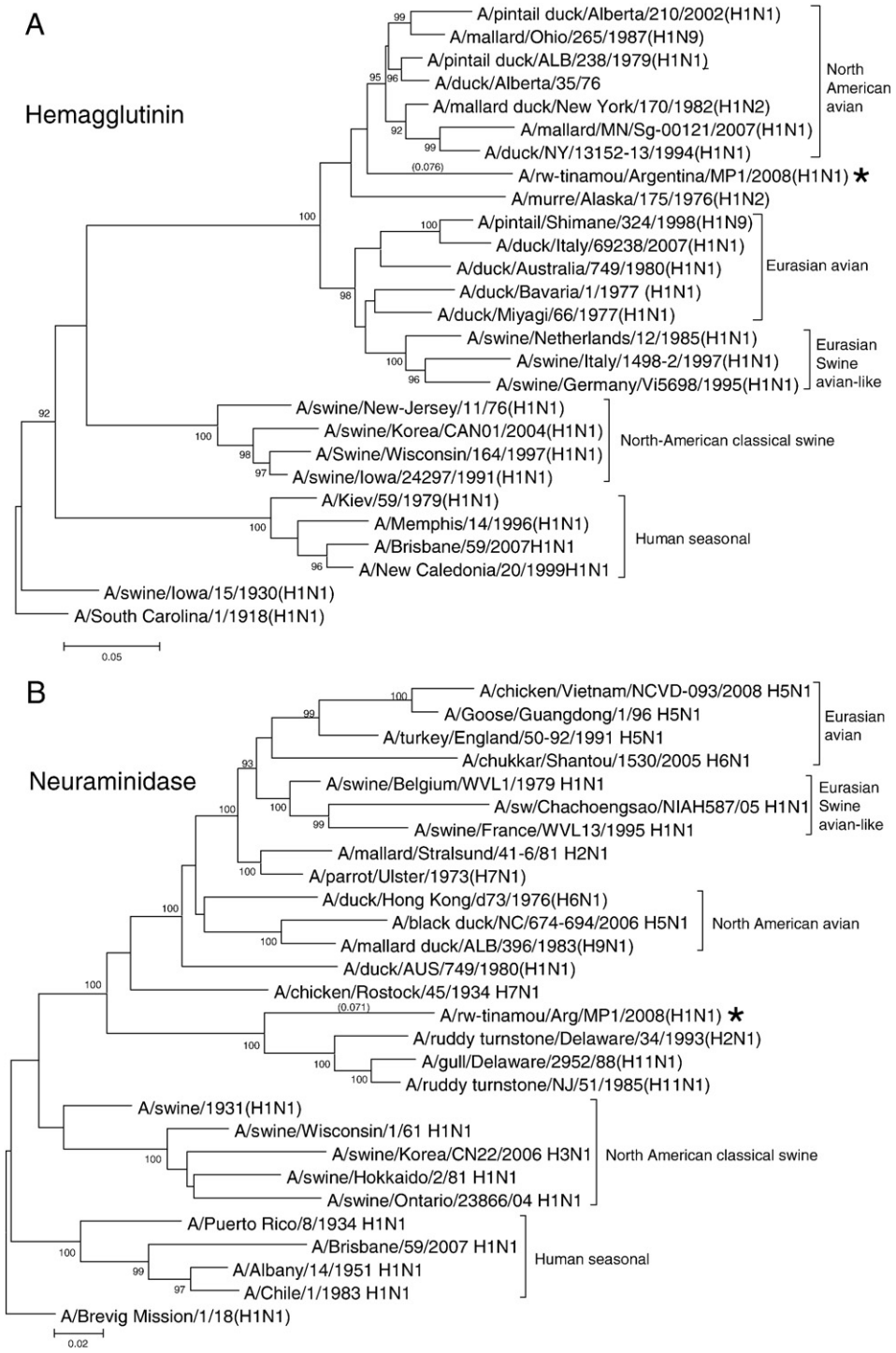

Fig. 1. Neighbor-joining phylogenetic trees of the HA (A) and NA (B) genes. The reliability of the phylogeny was assessed with 1000 bootstrap replicates. Values greater than 90% are indicated at the node of the branches. A/red-winged tinamou/Argentina/MP1/2008 genes are indicated with an asterisk (*). The horizontal bar denotes nucleotide substitutions per site. Length of the branches corresponding to the evolutionary path from tinamou virus gene to its sister cluster, is indicated in parentheses. The trees were rooted with 1918 viruses.

Phylogenetic analysis

Phylogenetic analysis showed that the tinamou virus H1 gene is closely related to the North American avian gene lineage (Fig. 1A). However, the long branch length corresponding to the tinamou sequence revealed a significant nucleotide divergence from the North American avian virus sequences. Furthermore, the tinamou isolate is the only representative virus from the South Hemisphere, suggesting incomplete influenza virologic surveillance in South American bird populations. Analysis of the tinamou H1 amino acid sequence showed that the cleavage site included only one basic amino acid, the typical structure of LP AIV. In contrast to all other H1 cleavage sites, alanine (A, hydrophobic amino acid, VPSIQ***A***R/GLF) was found preceding arginine, instead of a serine (S, polar non-charged amino acid, VPSIQ***S***R/GLF). The significance of this substitution is unknown.

Phylogenetic analysis revealed that the tinamou N1 gene is closely related to a limited set of North American avian viruses isolated from ruddy turnstones and gulls from New Jersey and Delaware in the 80's and 90's (Fig. 1B). As with the HA gene, the long branch corresponding to the N1 tinamou sequence and the fact that the tinamou isolate is the only isolate from the South Hemisphere suggest a gap in surveillance of the South American bird population.

In contrast to HA and NA, the tinamou virus internal genes are phylogenetically related to genes from South American isolates, as demonstrated by tree phylogeny (Figs. 2 to 5), where except for NS, all internal genes of South American isolates are tightly clustered. The nucleotide sequence of the internal genes of the tinamou virus revealed a close genetic similarity with sequences of viruses previously isolated in South America, namely A/kelp gull/Argentina/LCD4/06 (H13N9), A/cinnamon teal/Bolivia/4537/01 (H7N3) and A/chicken/Chile/4957/02(H7N3). The nucleotide identity between these three viruses and the tinamou isolate ranges from 85 to 97 %, except for NS, which shows more divergence than the other internal genes compared to the three South American viruses (69 to 97%, Supplementary Table 1).

The relationship between South American influenza gene lineages and other lineages from elsewhere in the world varies depending on the gene. The long branch lengths corresponding to the South American PB2 genes reveal a significant divergence from both North American and Eurasian avian lineages (Fig. 2). In contrast to PB2, the PB1 and M genes of viruses from South American lineages are closely related to viruses belonging to the North American lineage. This is supported by phylogenetic trees (Figs. 3A and B).

Phylogenetic analysis revealed that all the South American NS genes do not appear to share a single common ancestor. The tinamou NS gene, together with Chilean isolates are related to NS genes of allele B, whereas the other South American isolates like A/kelp-gull/Argentina/LDC4/2006 (H13N9) and A/cinnamon-teal/Bolivia/4537/2001 (H7N3) have an NS gene of allele A (Fig. 4). Finally, PA and NP genes of A/red-winged tinamou/Argentina/MP1/2008 (H1N1) are closely related to other isolates of South American lineage (Fig. 5). Interestingly, the phylogeny analysis revealed that both PA and NP genes of South American lineage share a common ancestor with the corresponding equine gene lineages (Fig. 5).

Discussion

Avian influenza virus was isolated from wild red-winged tinamous in Buenos Aires Province, in response to a call to investigate a die-off in this species in a hunting ground. Initially, the clinical symptoms were confused with an acute intoxication but the histopathological lesions were associated with viral infection. Influenza virus was readily detectable by RT-PCR (Supplementary Fig. 6). An influenza virus was subsequently isolated in embryonated chicken and duck eggs, characterized as type A, subtype H1N1, and denominated A/red-winged tinamou/Argentina/MP1/2008 (H1N1). The origin of the AI virus could not be determined, but it might be connected to free-ranging waterfowl attracted to the lakes during droughts. Coprophagia is common in red-winged tinamous and infected birds shed AIV in feces facilitating the rapid transmission of the virus through susceptible birds.

Following the identification and isolation of the AIV, a follow-up virologic and serologic survey was conducted in the area where the tinamou die-off took place 60 days earlier. Serum, cloacal and oropharyngeal swabs from captured red-winged tinamous and several other bird species were analyzed to detect AIV or antibodies

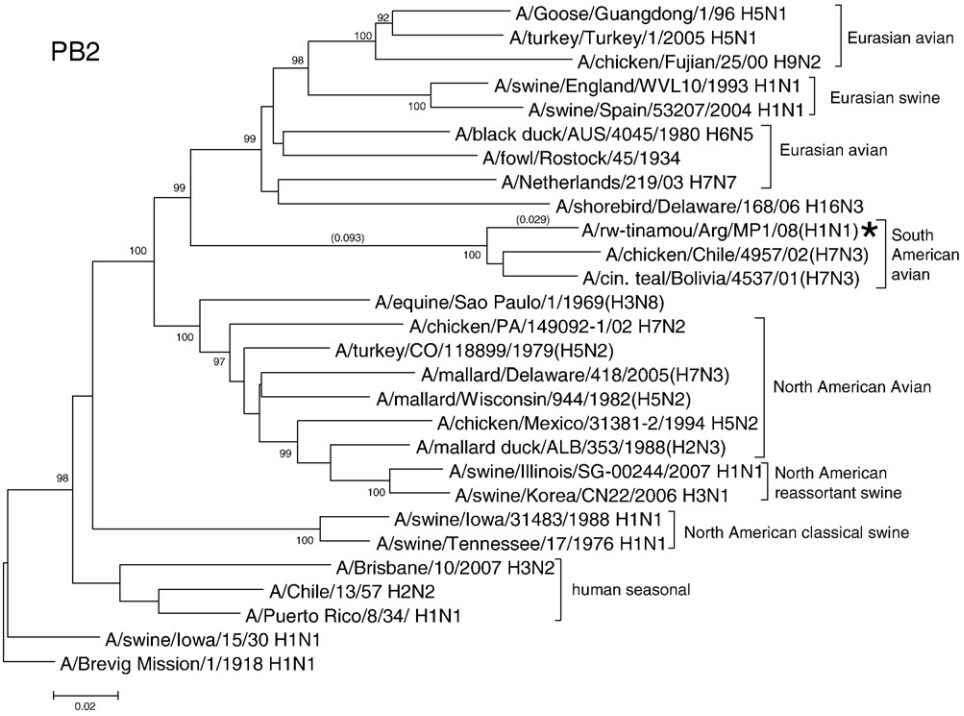

Fig. 2. Neighbor-joining phylogenetic tree of PB2 gene. Phylogeny reliability and notations are as in Fig. 1.

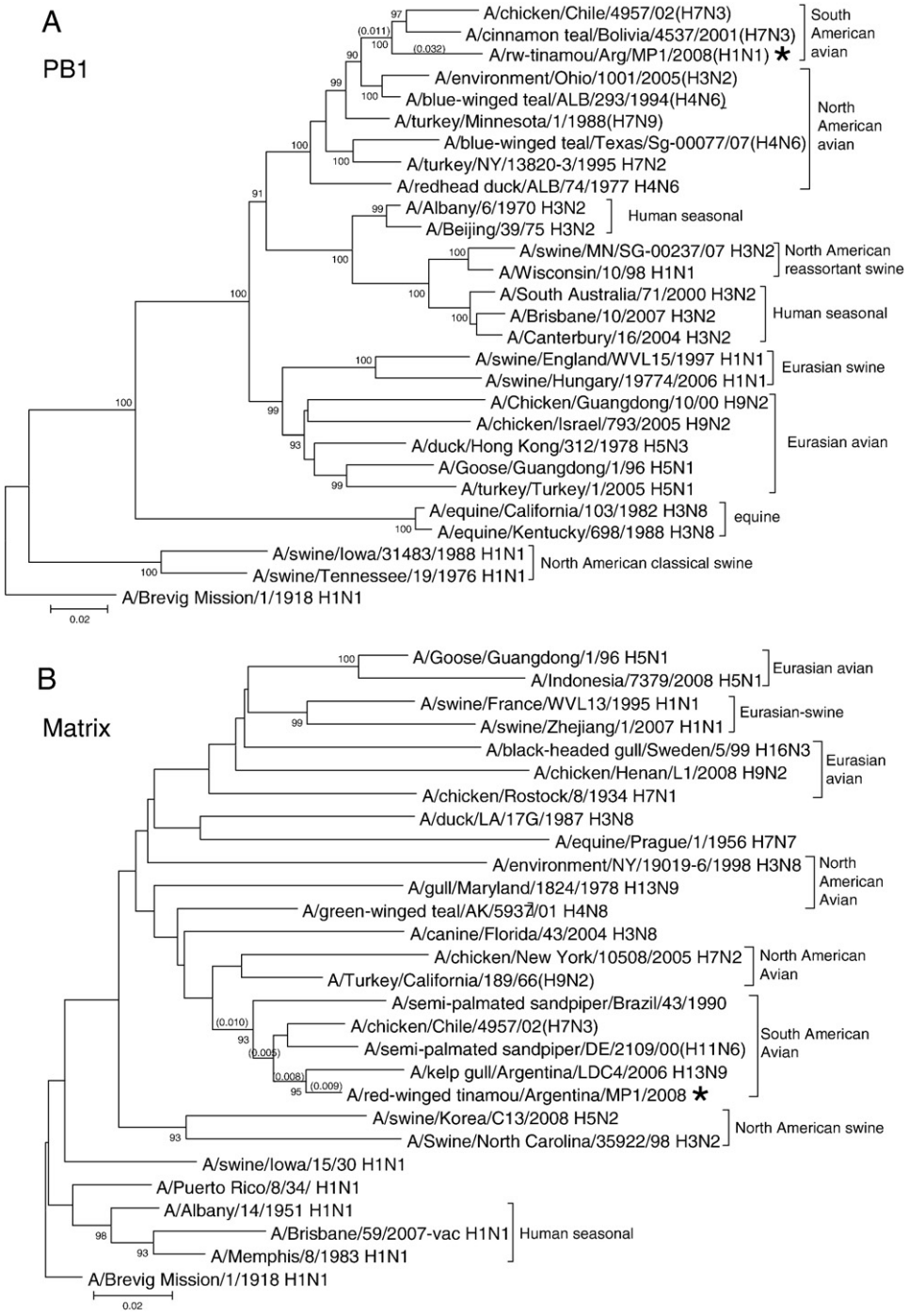

Fig. 3. Neighbor-joining phylogenetic trees of PB1 (A) and M (B) genes. Phylogeny reliability and notations are as in Fig. 1.

to it. Although virus could not be detected at this time, a high proportion of red-winged tinamous showed antibodies to the homologous virus, A/red-winged tinamou/Argentina/MP1/2008 (H1N1) in HI assays. In contrast, birds belonging to six other species captured in the hunting ground, as well as tinamous from a neighbor breeder, did not have detectable virus or antibody titers (Table 1). The same negative results were obtained in an official extended survey performed by SENASA, suggesting absence of circulation of AI viruses outside the hunting ground.

Red-winged tinamous appeared to be the only infected bird species in this case, as it follows from RT-PCR and serology assays performed in birds in the same area. Wetlands and surroundings are used for game bird hunting focusing mainly on ducks and red-winged tinamous. As the red-winged tinamou population has been decreasing in the last decades, it is also tempting to speculate that most of the birds found free in nature might be originated from irregular breeders that occasionally release birds for repopulation or game hunting purposes. Interspecies transmission events of IV are not rare, and in this regard it is known that although morbidity and mortality are rarely seen in the aquatic bird reservoir, they may occur when the virus enters another host species (Webster et al., 1992). Moreover, in this case, the stress generated by the change of habitat of the released birds might have rendered tinamous more susceptible to disease and mortality.

Red-winged tinamous are resident wild birds that inhabit grazing land and do not migrate. It remains unclear whether the influenza A virus was originated from the wild ecosystem or from agricultural

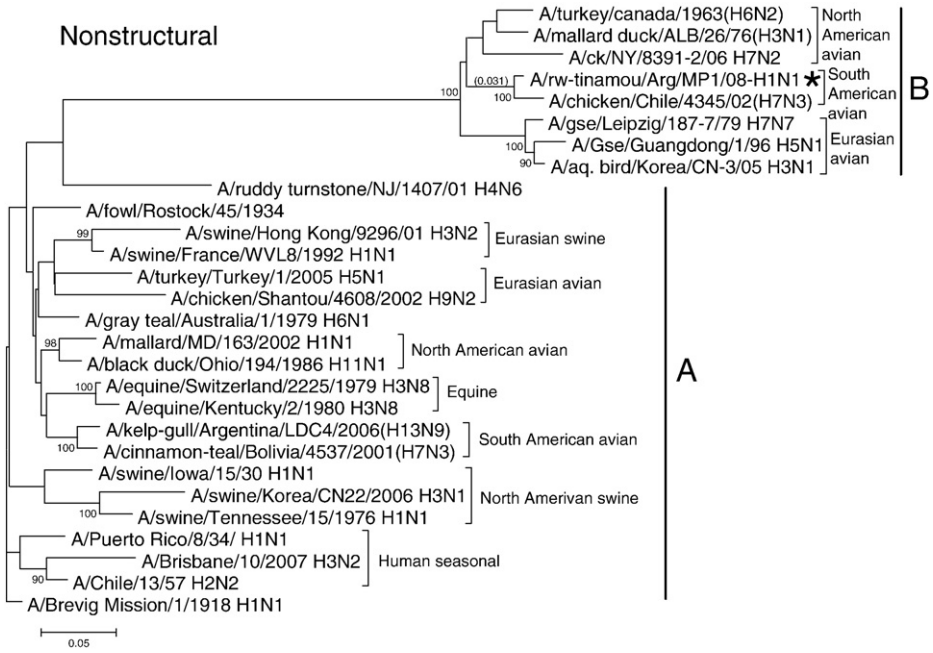

Fig. 4. Neighbor-joining phylogenetic tree of NS gene. Phylogeny reliability and notations are as in Fig. 1. Genes of alleles A and B are indicated.

sources, but the later is unlikely since influenza viruses have never been found in poultry in Argentina. On the other hand, as described in the Introduction section, 12 samples positive for influenza A virus had been detected in the past by RT-PCR in waterfowl and shorebirds, with only one isolation, subtyped as H13N9 (Pereda et al., 2008). In the tinamou case, we favor the hypothesis of a recent transmission from unidentified aquatic birds. We cannot absolutely rule out that the birds were just carriers of AIV and might die from other unidentified cause. However, the histopathological analyses were compatible with a viral infection, and the dead or sick birds were positive for AI, while potential breeder parents from whom the released birds might be originated were all negative (Table 1 and data not shown). In this sense, it appears that the tinamous acted as sentinels for the detection of AIV in the wild environment.

It is worth mentioning that the deduced amino acid sequence of the HA cleavage site of A/red-winged tinamou/Argentina/MP1/2008 (H1N1) did not correspond to a HPAI, but differed from any subtype H1 HA sequences previously reported in the presence of the an alanine instead of a serine preceding the arginine. The biological significance of this change is unknown and will be analyzed in future studies.

Surveillance in free-flying and wild land-based birds has been limited in South America and this fact is clearly reflected in the gaps shown in the phylogenetic trees constructed for HA and NA genes. The absence of other H1N1 isolates from South America, due most likely to gaps in virologic surveillance, leads to large evolutionary distances and limits our ability to reconstruct the evolution of H1 and N1 genes of the tinamou virus. Nevertheless, the findings of this work suggest that the evolutionary pathways of HA and NA genes related to the tinamou isolate involve exchanges between viruses circulating in the Northern and Southern hemispheres.

The presence of an avian South American lineage shared by isolates from Argentina, Chile, and Bolivia can be clearly identified for all internal genes, and strongly supports the postulated South American phylogenetic lineage for AIV PB2, PB1, PA, NP, M and NS genes (Pereda et al., 2008, Spackman et al., 2006, Suarez et al., 2004). Interestingly, the phylogeny of PB1 and M genes also revealed gene exchanges between North and South American lineages in recent time (Fig. 3), indicating the likely co-circulation of some North and South American viruses in migratory bird populations. The phylogenies of the remaining internal genes of the tinamou virus, as well as other South American viruses, also indicate relatively recent common ancestors to previously described North American avian internal gene lineages (Supplemental Fig. 7A–H). However, as has been previously described for other South American AIVs (Spackman et al., 2006), the genetic divergence between the two lineages is indicative of independent viral evolution and, presumably, independent circulation of distinct virus populations among South and North American birds.

Although influenza virus has been previously isolated from farm reared species of ratites, from rhea [A/Rhea/North Carolina/39482/93; Suarez et al., 1999], struthio [A/ostrich/Zimbabwe/222/96; Banks et al., 2000], and dromaius [A/emu/Texas/39442/93; Perdue et al., 1996], this is the first report of AIV isolation from land-based non-migratory wild birds in South America and the first isolate from the Tinamiformes order worldwide. This work may contribute to the knowledge about the origin and distribution of AI virus subtypes circulating in South America and to study the emergence of new viral variants that may cross species barriers, especially considering that surveillance in free-flying birds has been limited in this region.

Materials and methods

Sample collection and histopathological analysis

A cluster of respiratory disease among tinamou was found in a hunting ground constituted by approximately 5 km^2 of wetlands, which included several shallow lakes and the Durazno Creek area. The site is located in the Northwest area of Marcos Paz district (S 34° 52' 89" latitude and W 58° 52' 89" longitude), a rural area with predominantly animal farming (cattle, swine and poultry), based on natural prairie pastures located at 50 km Southwest of Buenos Aires City, Argentina (Supplementary Figs. 1 and 2).

Lungs, liver, kidneys, pancreas and spleen were collected from 58 red-winged tinamous found recently dead, and tracheal or cloacal swabs were taken from twelve live moribund birds, in the second week of April 2008. Specimens were stored at −70 °C until used for virus isolation. Tissue samples from lungs, liver, kidney, spleen, intestine and pancreas were fixed in 10% neutral-buffered formalin solution, routinely processed and stained with hematoxylin and eosin (HE) for histopathologic examination.

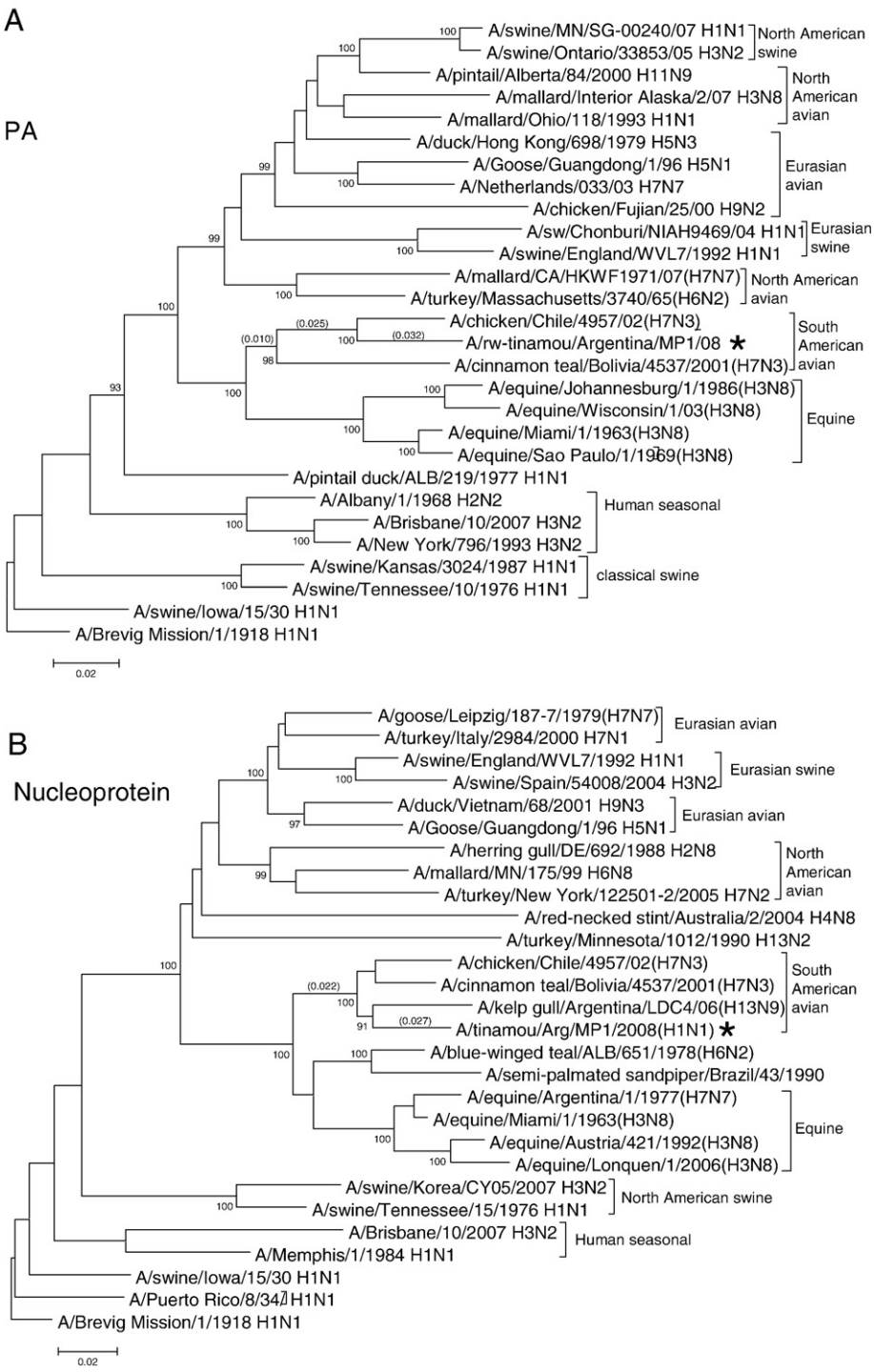

Fig. 5. Neighbor-joining phylogenetic trees of PA (A) and NP (B) genes. Phylogeny reliability and notations are as in Fig. 1.

A virologic and serologic survey of free-ranging birds in the same area was carried out two months later (June 2008). Cloacal and tracheal swabs and blood samples were collected from nineteen tinamous and five live-captured aquatic birds of each of the following species: white-faced tree-ducks (*Dendrocygna viduata*), brown pintails (*Anas georgica*), speckled teals (*Anas flavirostris*), white-winged coots (*Fulica leucoptera*), white-cheeked pintails (*Anas bahamensis*), and coscoroba swans (*Coscoroba coscoroba*). Five tinamous from an irregular breeder that occasionally released birds in the hunting ground were also sampled. Blood was collected from the jugular or ulna vein for serum separation. Cloacal and tracheal specimens were collected with Dacron swabs, singly resuspended in viral transport medium, transported with refrigeration and frozen at −50 °C on the same day.

An official survey was also conducted in an area of 10 Km radius from the location of the hunting ground by the Argentine Animal Health Service (SENASA), including backyard poultry.

AIV detection, isolation and characterization

Tissues were thawed and homogenized in phosphate-buffered saline (PBS) supplemented with 50 μg/ml of gentamicin and clarified by low-speed centrifugation. Pools of liver or lung homogenates (0. 1 ml) from red-winged tinamous were inoculated into

the allantoic cavity of ten 10-day-old embryonated chicken and duck eggs, as described previously (WHO, 2005). Presence of virus in the allantoic fluids was subsequently determined by hemagglutination (see below). Allantoic fluids with hemagglutinating activity were also tested using a hemagglutination inhibition (HI) test with polyclonal antibodies against La Sota strain of group 1 Newcastle disease virus (NDV).

Viral RNA was extracted from 250 µl of cloacal or tracheal swab eluates, organ homogenates or embryonated egg chorioallantoic fluids, using Trizol LS reagent (Life Technologies, Carlsbad, CA), in accordance with the manufacturer's instructions. RNA was eluted in a final volume of 100 µl and stored at −80 °C. cDNA synthesis and PCR were performed using One Step RT-PCR Kit (Qiagen®, Valencia, CA). Reverse transcription-PCR (RT-PCR) was performed as previously reported, with primers targeting a conserved HA2 region: HA-1134F/Em-NS-890R (Phipps et al., 2004). Amplification of a 640-pb fragment was expected for any of the 16 HA subtypes of type A influenza viruses.

PCR with primers specific for subtype N1 (WHO, 2005), which amplify a fragment of 616 bp, were also performed, using the viral strain A/New Caledonia/20/99 (H1N1) (origin World Health Organization) as positive control. The sequence of the primer pair was as follows: N1-1: 5′-TTGCTTGGTCGGCAAGTGC-3′; N1-2: 5′-CCAGTCCACCCATTTGGATCC-3′.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests

Virus-containing allantoic fluids were serially 2-fold diluted into U-bottom 96 well microtiter plates in a final volume of 50 µl. Freshly prepared 0.5% chicken red blood cells (cRBCs) were added and the plates were mixed by agitation, covered and allowed to settle at 4 °C for 2 h. The HA titer was expressed as the reciprocal of the last dilution which contained agglutinated cRBCs.

HI assays were conducted on the collected serum samples as reported (WHO, 2005). Briefly, serum samples were serially diluted 2-fold into U-bottom 96 well microtiter plates and an equal volume of the H1N1 isolated virus (6 HA units/50 µl) was added to each well. After incubation, freshly prepared cRBCs were added. The HI titer was expressed as the reciprocal of the last dilution that contained non-agglutinated cRBCs.

Gene sequencing and phylogenetic analysis

Genomic RNA corresponding to the 8 viral genes was amplified by RT-PCR as described by Hoffman et al. (2001), and PCR amplicons were sequenced directly with the BigDye terminator kit (Applied Biosystems™, Foster City, CA, USA) on an ABI 3730 (Applied Biosystems™, Foster City, CA, USA). Sequences were downloaded from NCBI Influenza Virus Resource (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). Multiple sequence alignments were generated by MAFFT software (http://align.bmr.kyushu-u.ac.jp/mafft/software/) (Katoh et al., 2002) and edited using BioEdit tool (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The dataset was curated, avoiding duplicates, frameshifts and short sequences (<90 % of full length). Successive phylogenetic trees were built with Mega4 using neighbor joining method and maximum composite likelihood model (Kumar et al., 2004) to identify redundant sequences and reduce the dataset. Once the dataset was manageable with Mega (~3000 sequences), gene lineages were identified and tinamou sequences were added for the analysis. Large phylogenetic trees are reported in supplementary figures. Phylogenetic tree reliability was estimated with 1000 bootstrap replications.

The nucleotide sequences of the 8 genes of A/red-winged tinamou/Argentina/MP1/2008 (H1N1) reported in this work were deposited in GenBank under the following accession numbers: PB2: GQ369462; PB1: GQ385248; PA: GQ379899; HA: GQ168615; NP: GQ168616; NA: GQ143810; M: GQ223719; NS: GQ202688.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.10.009.

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