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Isolation and characterization of an H9N2 influenza virus isolated in Argentina

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ABSTRACT

As part of our ongoing efforts on animal influenza surveillance in Argentina, an H9N2 virus was isolated from a wild aquatic bird (*Netta peposaca*), A/rosy-billed pochard/Argentina/CIP051-559/2007 (H9N2) – herein referred to as 559/H9N2. Due to the important role that H9N2 viruses play in the ecology of influenza in nature, the 559/H9N2 isolate was characterized molecularly and biologically. Phylogenetic analysis of the HA gene revealed that the 559/H9N2 virus maintained an independent evolutionary pathway and shared a sister-group relationship with North American viruses, suggesting a common ancestor. The rest of the genome segments clustered with viruses from South America. Experimental inoculation of the 559/H9N2 in chickens and quail revealed efficient replication and transmission only in quail. Our results add to the notion of the unique evolutionary trend of avian influenza viruses in South America. Our study increases our understanding of H9N2 viruses in nature and emphasizes the importance of expanding animal influenza surveillance efforts to better define the ecology of influenza viruses at a global scale.

1. Introduction

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae*. IAVs have in common 8 segments of genomic negative sense RNA. Wild aquatic birds are considered the natural reservoir of these viruses. Avian influenza viruses responsible for outbreaks in avian and mammalian species, including humans, are thought to evolve from wild bird strains. Two major lineages for avian influenza viruses have been defined, the Eurasian and North American lineages, respectively. Surveillance studies in Australia and South America suggest the presence of sub-lineages that evolve independently from other influenza genes in other regions. Avian influenza viruses from Australia appear to form a monophyletic lineage, whereas those from South American show independent evolution but share a common ancestor with North American viruses. Antigenic differences on the two viral surface proteins, hemagglutinin (HA) and neuraminidase (NA) have led to the classification of IAVs into subtypes; 16 HA and 9 NA subtype viruses have been described so far in wild aquatic birds (Bulach et al., 2010; Gonzalez-Reiche et al., 2012; Hansbro et al., 2010; Rimondi et al., 2011; Webster et al., 1992).

The H9N2 IAV subtype was first detected in the USA in 1966 (Homme and Easterday, 1970). In the late 1960s to early 1970s, H9N2 viruses were associated with a number of outbreaks in free-range turkeys in Minnesota, with viruses originating in wild ducks. To date, H9N2 influenza viruses are isolated sporadically from wild birds in North America (Halvorson et al., 1997; Kawaoka et al., 1988; Sharp et al., 1993, 1997). In Asia, H9N2 viruses were found in domestic ducks until the late 1980s before they crossed to domestic land-based poultry (Shortridge, 1992). Since then, H9N2s have caused disease outbreaks in poultry in a vast number of regions from South East Asia to the Middle East (Dong et al., 2011; Guo et al., 2000). China, Pakistan, Israel, Iran, and South Korea, among others, have reported H9N2 viruses in local live poultry markets where these viruses remain endemic (Fusaro et al., 2011; Lee et al., 2007; Naeem et al., 2007; Xu et al., 2007). More importantly, H9N2 viruses have been frequently transmitted to pigs, considered an intermediate host in the generation of influenza strains with pandemic potential (Peiris et al., 2001; Yu et al., 2011). Human infections

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with avian H9N2 viruses have also been reported and associated with mild influenza-like symptoms (Butt et al., 2005; Cheng et al., 2011; Lin et al., 2000). Serological studies suggest significant human exposure to these viruses in regions where they remain endemic (Khuntirat et al., 2011; Wang et al., 2009). Although no evidence of human-to-human transmission of H9N2 viruses has been observed, some H9N2 viruses circulating in poultry have evolved human-like receptor specificity and thus recognize sialic acids bound to the adjacent galactose in an α2,6 conformation (SAα2,6) (Matrosovich et al., 2001). Thus, the World Health Organization (WHO) considers H9N2 viruses as potentially pandemic strains (Capua and Alexander, 2002).

In contrast, no information regarding the presence of H9N2 viruses in South America is available. In this report, a H9N2 virus from an aquatic bird (559/H9N2) was isolated and characterized. Like other IAVs isolated in Argentina, the 559/H9N2 possesses a gene constellation consistent with a unique evolutionary group of IAVs from South America. Animal studies revealed limited replication with lack of transmission of 559/H9N2 in chickens, whereas in Japanese quail, efficient replication and transmission was observed. These results are consistent with previous studies showing that quail are more susceptible than chickens to H9N2 viruses isolated from ducks (Hossain et al., 2008; Makarova et al., 2003; Perez et al., 2003).

2. Materials and methods

2.1. Sample collection

Sampling activities were performed by trained biologists and veterinarians in the Lower Paraná River Valley. This valley is composed of a mosaic of rice fields, natural wetlands and marshes, native forests, and patches of land within the floodplain of the Parana River (30°41′S, 60°02′W) (Rimondi et al., 2011). Cloacal swabs were collected from carcasses of hunter-killed ducks donated by licensed hunters during the hunting seasons (April 15th to August 15th) of 2007 through 2008. Cloacal swabs were collected using single-use polyester sterile swabs and then stored separately in single plastic cryo-vials, containing 2 ml of Phosphate Buffer Solution (PBS) with 50% glycerol and Penicillin 10,000 IU/ml, Streptomycin 5 mg/ml, Gentamicin Sulfate 1 mg/ml, Kanamycin sulfate 700 μg/ml and Anphotericin B 10 μg/ml (Sigma Chemical Co, St. Louis, MO, USA). Samples were frozen in liquid nitrogen and transported on dry ice. Once in the laboratory all samples were stored at −80 °C until processed for molecular diagnosis and virus isolation.

2.2. Virus detection

Viral RNA was extracted from 140 μl of suspension from cloacal swabs using a QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions. RNA was eluted in a final volume of 60 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 using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The cDNA was tested for avian influenza virus (AIV) by real-time reverse transcriptase PCR (RRT-PCR) using TaqMan Universal PCR Master Mix (Applied Biosystems) directed to the matrix (M) gene. This system detects all type A influenza viruses (Spackman et al., 2002). The PCR was performed on an ABI Prism 7500 SDS (Applied Biosystems).

2.3. Virus isolation

Swab samples positive by RRT-PCR were inoculated into 9–11 day old specific pathogen free (SPF) embryonated chicken eggs. Briefly, 200 μl of PBS suspension from the cloacal swabs was injected into the egg's allantoic cavity and the eggs were incubated for 72 h and harvested in accordance with standard protocols described in the WHO Manual on Animal Influenza Diagnosis and Surveillance (Webster et al., 2005). Virus titration was performed by egg infectious dose 50 (EID50) following a previously described method (Reed and Muench, 1938) and using the hemagglutination assay as the final readout (Webster et al., 2005).

2.4. Phylogenetic and molecular analysis

Viral RNA was extracted from infected allantoic fluid using an RNEasy Mini kit (Qiagen). Reverse transcription followed by PCR was performed using specific primers for each gene segment as described previously (Hoffmann et al., 2001). PCR products were purified with a QIAQuick PCR purification kit (Qiagen). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems) following the manufacturer's instructions. Phylogenetic analyses were performed using additional influenza virus sequence data available in GenBank. Sequences were assembled and edited with Lasergene 8.1 (DNASTAR); BioEdit 7 was used for alignment and residue analysis. Neighbor-joining (NJ) trees were constructed by using PAUP* 4.0. Estimates of the phylogenies were calculated by performing 1000 NJ bootstrap replicates. The TREEVIEW program, Version 1.6.6 was used for visualization and printing of phylogenetic trees.

2.5. Animal studies

2-Week old SPF White Leghorn chickens (Charles River Laboratories, Wilmington, MA) and 4-week old Japanese quail (*Coturnix coturnix*, B & D Game Farm) were used throughout the studies. Chickens ($n = 4$) or quail ($n = 4$) were inoculated intraocularly, intranasally, and intratracheally with 5×10^6 EID_{50} of virus. Transmission was monitored by bringing the directly inoculated birds in direct contact with naïve birds ($n = 4$ birds/group) at 1 day post-inoculation (dpi). Tracheal and cloacal swabs were collected at 1, 3, 5, and 7 dpi in 1 ml freezing media (50% glycerol in PBS containing 1% antibiotics) and stored at −80 °C until use for virus titration. Birds were observed daily for 14 days for signs of disease. Birds were monitored for appetite, activity, fecal output, and signs of distress including cyanosis of the tongue or legs, ruffled feathers and respiratory distress. Experiments were carried out under ABSL2+ conditions with investigators wearing appropriate protective equipment and compliant with protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland and adhered strictly to the Animal Welfare Act (AWA) regulations.

2.6. Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study are available from GenBank under accession numbers CY111587 to CY111594.

3. Results and discussion

As part of ongoing animal influenza surveillance efforts an H9N2 IAV strain was isolated. Samples were collected between May 2007 and February 2008 at different hunting lodges in the Lower Paraná River Valley (30°41′S, 60°02′W) in Argentina. Details on species and samples have been previously described (Rimondi et al., 2011). Briefly, 1395 cloacal swabs representing 23 different bird species were collected and tested for the presence IAVs. Of these, 738 samples were obtained from rosy-billed pochards (*Netta peposaca*) and one of these samples resulted in the isolation of a H9N2 strain: A/rosy-billed pochard/Argentina/CIP051-559/2007 (H9N2)