Virus Research 150 (2010) 119–128

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Surveillance and characterization of low pathogenic H5 avian influenza viruses isolated from wild migratory birds in Korea

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ARTICLE INFO

Article history:
Received 22 December 2009
Received in revised form 3 March 2010
Accepted 4 March 2010
Available online 21 March 2010

Keywords:
Influenza virus
Migratory birds
H5N2
Phylogenetic analysis
South Korea

ABSTRACT

Migratory waterfowls are the natural reservoir of influenza A viruses. However, interspecies transmission had occasionally caused outbreaks in various hosts including humans. To characterize the genetic origins of H5 avian influenza viruses isolated from migratory birds in South Korea, phylogenetic analysis were conducted. A total of 53 H5 viruses were isolated between October 2005 and November 2008. Full genetic characterization indicated that most of these viruses belong to the Eurasian-like avian lineage. However, some segments of the AB/Korea/W235/07 and the AB/Korea/W236/07 isolates were clustered with North American lineage viruses rather than those of the Eurasian lineage, suggesting the occurrence of reassortment between these two avian virus lineages. Phylogenetic analysis further demonstrated that the H5N2 and H5N3 virus isolates were of the low pathogenicity H5 phenotype. The H5 viruses appear to be antigenically similar to each other, but could be distinguished from a recent HPAI H5N1 (EM/Korea/W149/06) virus by hemagglutinin inhibition (HI) assays. Experimental inoculation of representative viruses indicated that certain isolates, particularly AB/Korea/W163/07 (H5N2), could be detected in trachea and lungs of chickens but none could be transmitted by direct contact. Furthermore, all of the viruses could be detected in mice lung without prior adaptation which is indicative of their pathogenic potential in a mammalian host. Overall, our results emphasize the important role that migratory birds play in the perpetuation, transport, and reassortment of avian influenza viruses stressing the need for continued surveillance of influenza virus activity in these avian populations.

1. Introduction

Influenza A viruses have caused three major pandemics in the twentieth century and have occasionally caused disease outbreaks in various hosts such as, humans, avian species, and some types of mammals. Certain H5 and H7 subtypes of influenza viruses have occasionally obtained high pathogenicity (Kawaoka et al., 1984; Horimoto et al., 1995; Garcia et al., 1996; Pasick et al., 2005), posing unpredictable threat to public health and economical impact in the poultry industry. Although influenza A virus is capable of infecting many kinds of hosts, wild aquatic birds (such as ducks, geese, gulls, and shorebirds) are believed to constitute the major natural reservoir for all virus subtypes (Webster et al., 1992; Alexander, 2000). These viruses are occasionally transmitted to other non-avian hosts establishing stable lineage of influenza viruses in novel hosts, with the possible emergence of highly pathogenic avian influenza (HPAI) viruses in commercial animals or humans (Webster et al., 1992; Horimoto and Kawaoka, 2001; Gill et al., 2006; Chen et al., 2006; Webby et al., 2000)

Based on our unpublished surveillance data of avian influenza viruses isolated from migratory wild birds of South Korea from 2005 through 2008, low pathogenic avian influenza (LPAI) viruses of subtype H5 was the most commonly found virus displaying an isolation rate of about 18.8%. Previous studies have documented outbreaks of LPAI H5 in domestic poultry of Italy which may have been derived from viruses harbored by migratory birds according to serological or genetic investigation (Donatelli et al., 2001; De Marco et al., 2005). In northern Europe, LPAI H5 viruses were suggested to be the precursor strains responsible for outbreaks of HPAI H5N2 in poultry (Munster et al., 2005). A recent report in South Korea indicated the presence of avian-like LPAI H5N2 viruses in a number of Korean swine (Lee et al., 2009). Moreover, GS/Guangdong/1/96, the putative precursor of HPAI H5N1 Hong Kong/156/97, was likely derived from an LPAI H5 virus in migratory birds based on phylogenetic studies (Duan et al., 2007) whereas an LPAI H5N2 North American lineage virus caused poultry outbreaks in nearby Japan (Okamatsu et al., 2007). Taken together, LPAI viruses of migratory

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doi:10.1016/j.virusres.2010.03.002

birds seem to provide a dynamic pool of diverse influenza viruses which are frequently introduced to domestic animals which may lead to acquisition of high pathogenesis.

This study was conducted in response to the growing concerns on the pathogenic potential of H5 viruses currently circulating in wild migratory birds. Our data represent the surveillance and phylogenetic characterization of recent LPAI H5 viruses extensively isolated from wild migratory birds of South Korea from October 2005 to November 2008. We report herewith that most isolates are closely related with previous Korean aquatic bird influenza viruses or isolates of neighboring countries such as China or Japan. However, some strains interestingly possessed genes which are phylogenetically closer to those of the North American lineage rather than the Eurasian lineage suggesting intercontinental exchange of gene segments.

2. Materials and methods

2.1. Influenza virus collection and isolation

A total of 282 wild bird fecal samples positive for avian influenza virus infection were collected from major wild bird migratory sites in South Korea starting from October 2005 through November 2008. Collection months as listed in Table 1 correspond to wintering season of migratory birds coming from different regions into the Korean Peninsula, therefore considered as high risk period.

The obtained samples were suspended in cell culture media containing antibiotic solution [penicillin G (2×10^6 U/l), polymyxin B (2×10^6 U/l), gentamicin (250 mg/l), nystatin (0.5×10^6 U/l), ofloxacin HCl (60 mg/l), and sulfamethoxazole (0.2 g/l)] and thoroughly mixed by vortexing. To prevent cross-contamination, sterile materials (including centrifuge tubes and tips) were used for processing the fecal samples. The mixture was centrifuged at 3000 rpm for 15 min and supernatants were transferred into a fresh 1.5 ml tube. This procedure was repeated two more times to remove fecal debris and the final clarified supernatant was inoculated in 10-day-old embryonated chicken eggs and incubated at 37 °C for 48 h. The presence of virus was detected by hemagglutination assays (HA test) performed according to WHO/World Organization for Animal Health recommendations. The allantoic fluid from positive samples were harvested and stored at −80 °C until use. Subtyping was done by multiplex reverse transcription (RT)-PCR assays and confirmed by sequencing as previously described (Chang et al., 2008; Pascua et al., 2008). Positive samples were also validated by re-amplification using subtype-specific primers.

2.2. Genetic and phylogenetic analyses

Viral RNAs were isolated by using RNeasy mini kit (Qiagen, Valencia, CA) as recommended by the manufacturer's instructions. The extracted RNA was reverse-transcribed at 37 °C for 60 min using Omniscript RT kit (Qiagen, Valencia, CA). The synthesized cDNA were amplified by PCR reactions which were performed using Ex *Taq* polymerase (TAKARA, Shiga, Japan) according to the manufacturer's protocol. Then, the amplicons were extracted and purified with a QIAquick gel extraction kit (QIAGEN) and sequenced at Cosmogenetech (Seoul, South Korea) with an ABI 373 XL DNA sequencer (Applied Biosystems, Foster City, CA). DNA sequences were edited and aligned using the Lasergene sequence analysis software package (DNASTAR, Madison, WI). Sequences were aligned by the CLUSTAL_V (Higgins et al., 1992) program and phylogenetic trees were constructed using full-length nucleotide sequences from this study and sequences available from GenBank database. Constructed trees were prepared by using the neighbor-joining algorithm and further plotted by using NJ Plot (Perriere and Gouy, 1996). Branch lengths are proportional to sequence divergence and can be measured relatively to the scale bar included in the figures. Numbers at the branches indicate the branch stability over 1000 bootstrap replicates. Complete sequences of all eight genes of the representative viruses (isolated from 2005 to 2008) and previously reported wild bird and domestic Korean H3 viruses (Song et al., 2008) were added to the phylogenetic analyses.

Table 1
Number of avian influenza virus isolates, October 2005–November 2008.

Years and months	No. of positive samples	No. of AIV isolates positive for subtype					
		H1 (%)	H3 (%)	H5 (%)	H7 (%)	H9 (%)	Others (%)[a]
2005							
October	13	0	1 (7.7)[b]	1 (7.7)	0	2 (15.38)	9 (69.2)
November	8	0	0	2 (25)	1 (12.5)	1 (12.5)	4 (50)
December	7	0	0	3 (42.9)	1 (14.3)	0	3 (42.9)
2006							
January	1	0	0	1 (100)	0	0	0
February	2	0	0	0	2 (100)	0	0
March	1	0	1 (100)	0	0	0	0
October	1	0	1 (100)	0	0	0	0
November	18	0	1 (5.6)	5 (27.8)	0	0	12 (66.7)
December	27	0	0	3 (11.1)	6 (22.2)	0	18 (66.7)
2007							
January	24	0	0	3 (12.5)	2 (8.3)	0	19 (79.2)
February	10	0	0	0	0	0	10 (100)
October	8	0	1 (12.5)	3 (37.5)	0	0	4 (50)
November	40	4 (10)	0	8 (20)	1 (2.5)	3 (7.5)	24 (60)
December	61	1 (1.6)	0	17 (27.9)	0	0	43 (70.5)
2008							
January	26	0	0	3 (11.5)	1 (3.8)	0	22 (84.6)
February	2	0	0	2 (100)	0	0	0
April	3	0	0	0	1 (33.3)	1 (33.3)	19 (33.3)
November	30	3 (10)	2 (6.7)	2 (6.7)	0	0	23 (76.7)
Total	282	8 (2.8)	7 (2.5)	53 (18.8)	15 (5.3)	7 (2.48)	192 (68.1)

[a] Other HA subtypes detected which include H2, H4, H6, H8, H10, H11 and H12.
[b] Percentage distribution of monthly H5 positive isolates over the total number of positive samples detected by RT-PCR and sequence analysis (Chang et al., 2008).