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Research Article

Genetics and biological property analysis of Korea lineage of influenza A H9N2 viruses

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ABSTRACT

H9N2 influenza viruses have been detected from wild and domestic avian species including chickens and ducks worldwide. Few studies have compared the biological properties of different H9N2 lineages or determined whether certain lineages might pose a higher risk to mammals, especially H9N2 viruses of Korean lineage. The objective of this study was to characterize the genetic and biological properties of 22 Korean H9N2 viruses and assess their potential risks to mammals. Their complete genomes were analyzed. Some Korean H9N2 viruses were found to carry mammalian host-specific mutations. Based on genomic diversities, these H9N2 viruses were divided into 12 genotypes. All 22 showed preferential binding to human-like receptor. Two of eight H9N2 viruses were highly lethal to mice, causing 90–100% mortality without prior adaptation and severe respiratory syndromes associated with diffuse lung injury, severe pneumonia, and alveolar damage. These findings suggest that recent Korean H9N2 viruses might have established a stable sublineage with enhanced pathogenicity to mice. Various H9N2 strains pathogenic to mice were endemic in wild bird, poultry farm, and live bird markets, suggesting that Korean H9N2 viruses could evolve to become a threat to humans. The findings emphasize the necessity of careful, continuous, and thorough surveillance paired with risk-assessment for circulating H9N2 influenza viruses.

1. Introduction

Influenza A viruses (Influenza virus A genus, Orthomyxoviridae family) have a broad spectrum of hosts including birds, pigs, humans, and several other mammals (Krumbholz et al., 2014). The natural reservoir of influenza A viruses is wild waterfowl (Causey and Edwards, 2008). The antigenic and genetic diversities of two surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), have been used to classify type A influenza viruses into subtypes. A total of 18 HA and 11 NA subtypes have been reported for type A influenza viruses (Tong et al., 2013; Zhu et al., 2012). Avian influenza virus (AIV) has been studied increasingly over the last few decades, especially after H5N1 (a highly pathogenic avian influenza virus, HPAIV) outbreak in poultry (Hoye et al., 2010). The precursor of H5N1 has been thought to be low pathogenic avian influenza virus (LPAIV) that naturally circulates in wild bird populations (Alexander, 2007). Of the various LPAIV, H9N2 influenza viruses have been detected from wild and domestic avian species including chickens and ducks worldwide. Their low pathogenic nature to poultry has made them a low priority in animal disease control (Zhang et al., 2009). However, H9N2 viruses can cause human infections. Examinations in Asia have found serological evidence of human exposure to H9N2 viruses, suggesting the prevalence of mild or subclinical human infections of H9N2 viruses (Huang et al., 2013; Khan et al., 2015; Li et al., 2016; Sikkema et al., 2016; Uyeki et al., 2012; Wang et al., 2015). The source and risk factors for H9N2 transmission from poultry to human and the risk of infection by H9N2 viruses among persons working in live poultry markets where these viruses are prevalent are currently under investigation.

A recent phylogenetic classification of H9N2 viruses described 23 distinct clades with five clade-specific outliers (Shanmuganatham et al., 2016). The G1-like lineage represented by A/quail/Hong Kong/G1/1997 and the Y280-like lineage represented by A/chicken/Hong Kong/G9/1997 are the two most predominant progenitor lineages of H9N2 viruses currently circulating in China, Northeast Asia, and the Middle East (Group, 2013). They are routinely isolated from wild birds and occasionally from pigs and other mammalian species. Although H9N2 viruses are only endemic in poultry currently, they are the leading candidates with the potential to be transmitted to humans and cause lethal infections (Watanabe et al., 2014).

H9N2 viruses use domestic poultry as an intermediate host. They

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are predominantly isolated from live-bird markets, a proven risk factor for zoonotic transmission between birds and humans (Bi et al., 2010; Pawar et al., 2012; Peiris et al., 1999; Wang et al., 2015). H9N2 has contributed to the genesis of H5N1, a potential pandemic threat (Lin et al., 2000; Shanmuganatham et al., 2014). H9N2 viruses may have contributed six internal genes (PB2, PB1, PA, NP, M, and NS) to the newly emerged H7N9 virus in southern China, and to the H10N8 virus that has caused three human infections in Jiangxi province of China (Chen et al., 2014; Gao et al., 2013; Zhang et al., 2013). H9N2 reassortants bearing genes from the 2009 H1N1 pandemic virus exhibit higher virulence in mice or increased transmissibility in ferrets (Kimble et al., 2011).

These facts prompted us to assess the biologic properties and pandemic potential of H9N2 influenza viruses circulating in poultry. Surveillance studies from South Korea have shown that H9N2 viruses are endemic in chickens and quails, with live-bird markets serving as an epicenter for infection and transmission (Park et al., 2011). Since their introduction in late 1996, H9N2 viruses have evolved tremendously via intra- and inter-subtype reassortment with genetic drift. They have become unique and distinct from their prototype MS96 virus (A/Chicken/Korea/MS96/96) (Lee et al., 2016). However, few studies have compared the biological properties of H9N2 viruses of different lineages or determined whether certain lineage might pose higher risk to mammals, especially those of Korean lineage. Almost all studies have focused on G1 lineage viruses isolated from China. To fill the gap in knowledge, this study sought to extensive characterize South Korea H9N2 viruses and assess their risk.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict compliance with legal regulations of Laboratory Animals Act and Animal Welfare Act of South Korea. The protocols for animals studies and use of 10-day-old embryonated eggs were approved by the Institutional Animal Care and Use Committee (CBU 2014-00076) at Chonbuk National University. All experiments with live H9N2 viruses were conducted within an enhanced animal biosafety level 2+ (ABSL2+) facility. The animal isolators in the facility are hyperfiltered. The researchers who work with mice always wear N95 masks and disposable overalls.

2.2. Virus and 10-day-old embryonated eggs

To assess the biologic properties of H9N2 viruses and their risks to mammals, representative isolates (Table 1) were selected from our ongoing avian influenza surveillance. These H9N2 viruses were isolated from poultry in different regions of South Korea between 2002 and 2016. Virus stocks were grown in 10-day-old specific pathogen-free chicken eggs, which were purchased from BioPOA Co. (Yongin, Korea). Viral titers (EID_{50} values) were determined as described previously (Reed and Muench, 1938).

Table 1
Ten H9N2 viruses and two standard H1N1 viruses used in the experiment.

No.	Virus	Genotype	Strain (Abbreviation)
1	H1N1	–	A/NWS/33 (NWS/H1N1)
2		–	A/California/07/2009 (CA/pH1N1)
3	H9N2	A	A/chicken/Korea/MS96/96 (MS96)
4	Standard Classical	B	A/chicken/Korea/01310/2001 (01310)
5	H9N2	A	A/wild waterfowl/Korea/25/2008 (W25)
6	wildtype classical	B	A/chicken/Korea/SL-4P/2009 (SL-4P)
7		C	A/chicken/Korea/C9/2009 (C9)
8		C	A/chicken/Korea/C14/2009 (C14)
9		D	A/chicken/Korea/C16/2009 (C16)
10		D	A/chicken/Korea/C21/2009 (C21)
11		G	A/chicken/Korea/C54/2009 (C54)
12		J	A/wild waterfowl/Korea/7/2010 (W7)

2.3. Genetic and phylogenetic analysis

Viral gene amplification and sequencing were carried out as described previously (Hoffmann et al., 2001). Briefly, viral RNAs were extracted from virus stocks of these 22 H9N2 isolates using RNeasy Mini Kit (QIAGEN, Mississauga, ON, Canada). They were reverse transcribed to cDNAs using Omniscript Reverse Transcriotase kit (Qiagen, Hilden, Germany). PCR amplifications were carried out using influenza-specific primers and nTaq DNA polymerase. Nucleotide sequencing was performed using BigDye Terminator v3.1 Cycle sequencing Kit and ABI PRISM genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequence data were complied with SEQMAN program. Phylogenetic analyses were carried out using neighbor joining algorithm and plotted with NJPlot. Complete genome sequences of eight H9N2 viruses isolated from domestic poultry and wild birds were compared to previous Korean isolates of H9N2. The genome sequences of these 22 viruses reported in this study were deposited at GenBank (KY776425–KY776432, KY777702–KY777709, KY785726–KY785878).

2.4. Receptor binding analysis

Receptor specificity was analyzed using solid-phase direct binding assay with two different glycopolymers {α-2, 3-siaylglycopolymer [Neu5Acα2-3Galβ1-4GlcNAcβ1-pAP (*para*-aminophenyl)-alpha-polyglutamic acid (α-PGA)] and the α-2, 6-sialylglycopolymer [Neu5Acα2-6Galβ1-4GlcNAcβ1-pAP (*para*-aminophenyl)-alpha-polyglutamic acid (α-PGA)]} as described previously (Zhang et al., 2009; Zhu et al., 2012) with slight modifications. Briefly, viruses were grown in 10-day-old embryonated eggs (BioPOA Co., Yongin, Korea), clarified by low-speed centrifugation, laid over a cushion of 30% sucrose in phosphate buffered saline (PBS), and ultra-centrifuged at 28,000 r.p.m for 2 h at 4 °C. Virus stocks were aliquoted and stored at −80 °C until use. Virus titers were determined using haemagglutination assays with 0.5% chicken red blood cells (cRBCs) purchased from BioPOA Co. (Yongin, Korea). Microtitre plates (Nunc, Roskilde, Denmark) were incubated with two-fold serial dilutions of sodium salts of sialyglycopolymers in PBS at 4 °C for 30 min. These plates were then exposed to UV light (254 nm) for 10 min. After removing the glycopolymer solution, plates were washed three times with PBS. Then, 50 µl of virus suspension diluted in PBST (PBS containing 0.1% Twen-20) was added to each well and the plate was incubated at 4 °C for 2–3 h. After washing five times with 250 µl of PBST, plates were fixed with 10% formalin in PBST for 30 min. After fixing, plates were washed again five times with PBST and 50 µl of chicken antiserum against A/chicken/Korea/MS96/96 (H9N2) virus diluted in PBST was added to each well followed by incubation at 37 °C for 1 h. After washing five times with PBST, these plates were incubated with horseradish peroxidase (HRP)-conjugated goat-anti-chicken antibody (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 1 h. After washing with PBST five times, these plates were then incubated with *O*-phenylenediamine (Sigma-Aldrich) in PBS containing 0.01% hydrogen peroxide (H_2O_2) at room temperature for 10 min. The reaction was stopped by the addition of 50 µl of 0.5 M H_2SO_4. The optical density of each well at wavelength of 490 nm was then determined on a microplate reader (Perkin-Elmer, Rodgau, Germany). Dose-response curves of virus binding to glycopolymers were obtained using a single site binding algorithm and curve fitted using GraphPad Prism to determine the association constant values (Ka). The value of mean ± SD of three independent experiments was presented. Each sample was performed in triplicates in each experiment.