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Assessment of pathogenicity and antigenicity of American lineage influenza H5N2 viruses in Taiwan☆

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ABSTRACT

During December 2003 and March 2004, large scale epidemics of low-pathogenic avian influenza (LPAI) H5N2 occurred in poultry farms in central and southern Taiwan. Based on genomic analysis, these H5N2 viruses contain HA and NA genes of American-lineage H5N2 viruses and six internal genes from avian influenza A/H6N1 viruses endemic in poultry in Taiwan. After disappearing for several years, these novel influenza H5N2 viruses caused outbreaks in poultry farms again in 2008, 2010 and 2012, and have evolved into high pathogenic AI (HPAI) since 2010. Moreover, asymptomatic infections of influenza H5N2 were detected serologically in poultry workers in 2012. Therefore, we evaluated antigenicity and pathogenicity of the novel H5N2 viruses in ferrets. We found that no significant antigenic difference was detected among the novel H5N2 viruses isolated from 2003 to 2014 and the novel H5N2 viruses could cause mild infections in ferrets. Monitoring zoonotic transmission of the novel H5N2 viruses is necessary.

1. Introduction

Based on phylogenetic analysis of influenza HA genes, two lineages of avian influenza H5 viruses, Eurasian and American lineages, are circulating globally (Ip et al., 2015; Lee et al., 2014; Xu et al., 2015). The American-lineage avian influenza A/H5N2 caused avian outbreaks in the US in the 1980s and Mexico in 1990s (Horimoto et al., 1995). Since 1994, the American-lineage H5N2 viruses became endemic in Mexico and inactivated vaccines produced using the Mexico94-like viruses have been used in Mexico (Lee et al., 2014). In Taiwan, no H5 viruses had been detected before 2003 despite its high-density practice of poultry farming. During December 2003 and March 2004, large scale epidemics of low-pathogenic avian influenza (LPAI) H5N2 occurred in poultry farms in central and southern Taiwan (Cheng et al., 2010). These H5N2 viruses contain HA and NA genes of the America-lineage H5N2 viruses and six internal genes from avian influenza A/H6N1 viruses endemic in poultry in Taiwan. These reassortant H5N2 viruses were isolated in poultry farms again in 2008 and 2010 in which the predominant viruses drifted from LPAI into high-pathogenic avian influenza (HPAI). In February 2012, HPAI H5N2 outbreaks occurred in chicken farms in western Taiwan resulting in 8147 confirmed cases and 5497 fatal chickens (Cheng et al., 2010; Lee et al., 2014).

During the 2012 HPAI H5N2 outbreaks, Taiwan Centers for Disease Control (CDC) conducted clinical and serologic surveys on 141 subjects, including poultry workers, animal health officials, and temporary employees, who had close contacts with the infected animals. These subjects did not develop influenza-like illness within a week after the close contacts with chickens. Overall, this human study did not detect any virus shedding in throat swabs of these high-risk groups but found that 6 (4.3%) subjects had elevated antibody titers detected by using hemagglutination inhibition (HI) and neutralization assays (Wu et al., 2014). In addition, Taiwan CDC also conducted cross-sectional serosurveys in live poultry vendors, poultry farmers, and non-poultry workers in mid-2012. The seroprevalence of HI antibody against the American-lineage H5N2 in these three groups were 2.99% (10/335), 1.79% (6/335), and 0.35% (2/577), respectively (Huang et al., 2015). Overall, these serological results indicate the occurrence of subclinical infections of influenza H5N2 in poultry workers and could pose threats to public health. The ferret model of influenza infection provides important information to assess the

☆ Evaluation of influenza H5N2 reassortant viruses.

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potential of avian influenza viruses to infect humans and cause disease (Belser et al., 2011). Therefore, we evaluated antigenicity and pathogenicity of the novel American-lineage influenza H5N2 reassortant viruses in ferrets.

2. Materials and methods

2.1. Virus, Cell, and medium

All wild-type viruses used in the study were isolated and amplified in specific pathogen free (SPF) embryonated hens' eggs in Animal Health Research Institute (AHRI), Taiwan. The accession numbers of the Taiwan H5N2 viral genomes are provided in Supplementary Table 1. Three H5N1 reassortant vaccine viruses used in hemagglutination inhibition (HI) assay, NIBRG-14 (A/Vietnam/1194/2003×A/PR/8/1934), IDCDC-RG6 (A/Anhui/1/2005×A/PR/8/1934, and IDCDC-RG30 (A/Hubei/1/2010×A/PR/8/1934) were generated using reverse genetics and obtained from the National Institute for Biological Standards and Control (NIBSC) or the US Centers for Disease Prevention and Control (CDC) (Dong et al., 2009). Primary chicken embryonic fibroblasts (CEF) were generated from SPF embryonated chicken eggs. Briefly, embryos from 10-day-old eggs were ground with 2 μg/ML TPCK-trypsin (Sigma) in Petri-dishes and incubated in 37 °C for 10 min. After incubation, 5% fetal bovine serum was added to the homogeneous tissue mixture for trypsin neutralization. Tissue debris was discarded after centrifugation. The supernatant was cultured with Medium 199 (Gibco) plus 10% fetal bovine serum, and the medium were replaced next day to remove the unattached cells. All experiments handling HPAI viruses were conducted in Biosafety Level 3 laboratories in AHRI, Taiwan.

2.2. Phylogenetic analyses

Full sequences of influenza HA genes in the study were obtained from GISAID EpiFlu Database or Taiwan Animal Health Research Institute (AHRI). DNA sequences were compiled and analyzed using MEGA7 (Kumar et al., 2016). Multiple alignments were performed using the MUSCLE method. The phylogenetic tree was generated using maximum-likelihood (ML) inferred with General Time Reversible model. 1000 bootstraps replicates were conducted to evaluate the robustness of the ML topology.

2.3. Virus titration

Based on our pilot tests, the avian influenza H5N2 reassortant viruses could not grow well in mammalian cells (such as MDCK and Vero cells). Therefore, we selected CEF for conducting virus titration (Moresco et al., 2010). Infectious virus titers in tissues and nasal washes were determined in CEF using 50% tissue culture infectious doses (TCID50) assay. The homogenized tissues and nasal washes were serial diluted in DMEM and inoculated in 96-well microplates planted with CEF. After 1 h incubation, the supernatant was replaced with fresh serum-free medium. The plates were incubated at 35 °C for four days to record the cytopathic effect of the cells and supernatants were checked for hemagglutination ability to confirm the existence of influenza viruses. A positive control with pre-specified acceptable range was included to assure the test accuracy.

2.4. Quantitative RT-PCR to measure viral RNA

The amount of viral RNA in tissues and nasal washes were measured using RT-PCR as described previously (Chia et al., 2015). Briefly, one hundred milligrams of homogenized tissue sample was suspended in DMEM without serum. After centrifugation for removing debris, viral RNA was isolated by using the QIAamp Viral RNA Mini kit (Qiagen). Reverse-transcribed cDNA from RNA samples were obtained by using HiScript I Reverse Transcriptase (Bionovas) with Random Hexamer Primers (Bioline) according to the manufacturer's instructions. The RNA copy number of each cDNA samples was determined using a 7500 Real-Time PCR System (Applied Biosystems) with the primer targeting influenza Matrix (M) gene. Positive and negative reference samples were tested along with the unknown samples, and each sample was tested in duplicate.

2.5. Ferret experiments

4 to 6-month-old ferrets that were HI antibody seronegative to A/chicken/Taiwan/1209/2003 (H5N2) virus were bred and housed in Institute of Preventive Medicine, National Defense Medical Center. In addition, we also measured serum HI antibody to three seasonal influenza viruses, including A/Texas/89/2009 (H1N1), A/Wisconsin/67/2005 (H3N2), and B/Finland/214/2009. Only low serum HI antibody titers against the H3N2 virus were detected in these ferrets. Since there is no HI antibody cross-reactivity between the H3N2 and H5N2 viruses, the ferrets were evaluated to be suitable for this study. Before the animal study, the ferrets were implanted with microchips for measuring body temperature. During the experiments handling with influenza H5N2 viruses, ferrets were housed separately in an Animal Biosafety Level 3 (ABSL-3) facility. For assessment of viral antigenicity, two inoculations of each wild-type H5N2 virus (10^7 TCID50/0.5 ML/inoculation) were given intranasally to a group of two ferrets at Day 0 and Day 14. Sera from the inoculated ferrets were collected at days 0, 14, and 30 for measuring HI titers. For pathogenicity test, animals were anesthetized and inoculated intranasally with wild-type H5N2 influenza viruses in 10^8 TCID50/inoculation since no clinical sign was observed in ferrets inoculated with 10^7 TCID50 during the antigenicity test. Clinical signs were recorded daily for seven days post infection (DPI). At 3 and 7 DPI, tissues of several respiratory organs, including nasal turbinate, upper respiratory tract, lower respiratory tract, lung, and hilar lymph node, were homogenized for measuring viral RNA copies using quantitative RT-PCR (qPCR). Infectious virus titers were quantified using CEF cells and the TCID50 assay.

2.6. Hemagglutination inhibition assay

The HI titer of the ferret sera was measured using 0.5% chicken red blood cells (RBC) or turkey RBC reacting with 4 HA unit of inactivated virus antigen as described previously (Chia et al., 2015). HI titer was read according to the reciprocal of the highest dilution of the serum inhibiting hemagglutination. A positive control serum with a pre-specified acceptable range was included to confirm assay accuracy.

2.7. Ethics statement

All animal experiments were conducted according to the guidelines of Institutional Committee on Animal Care and Use, Institute of Preventive Medicine, National Defense Medical Center following the Institutional Animal Care Committee Guidebook published by the US Office of Laboratory Animal Welfare.

3. Results

We first conducted phylogenetic analysis to select representative viruses for animal experiments. Similar to the previous phylogenetic analysis of HA genes, the Taiwan H5N2 viruses isolated from 2003 to 2014 clustered with the American-lineage H5N2 viruses and were close to the Mexico vaccine strain virus, A/chicken/Hidalgo/28159-232/1994 (GenBank Accession: CY006040) (Fig. 1). The similarity of the HA protein sequences among the Taiwan H5N2 viruses was above 93% (Supplementary Figure 1), and neither of them possessed the mutations known to increase human receptor affinity, including N158D, N186K, Q226L, and S227N, in the receptor-binding pockets