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High frequency of reassortment after co-infection of chickens with the H4N6 and H9N2 influenza A viruses and the biological characteristics of the reassortants

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ABSTRACT

H4 and H9 avian influenza viruses (AIVs) are two of the most prevalent influenza viruses worldwide. The co-existence of H4 and H9 viruses in multiple avian species provides an opportunity for the generation of novel reassortants and for viral evolution. The diversity of the biological characteristics of the reassortants enhances the potential threat to the poultry industry and to public health. To evaluate the reassortment of these viruses and the potential public risk of the reassortants, we co-infected chickens with H4N6 and H9N2 viruses derived from poultry and tested the replication and virulence of the reassortant viruses in mice. A high frequency of reassortment was detected in chickens after co-infection with these two viruses and nine reassortants of six genotypes were purified from the chicken samples. Two H9N2 reassortants containing the PA of the parent H4N6 virus showed higher virulence than the parent H9N2 virus, revealing the significant role of the H4N6 *wt* virus PA gene in viral reassortment. Analysis of the polymerase activity of the ribonucleoprotein (RNP) complex in vitro suggested that the PA of H4N6 *wt* origin enhanced polymerase activity. Our results indicate that co-infection of an avian individual with the H4N6 and H9N2 viruses leads to a high frequency of reassortment and generates some reassortants that have higher virulence than the wild-type viruses in mammals. These results highlight the potential public risk of the avian influenza reassortants and the importance of surveillance of the co-existence of the H4N6 and H9N2 viruses in avian species and other animals.

1. Introduction

Influenza A viruses are segmented RNA viruses. Their genome is composed of eight single-stranded, negative-sense viral RNA segments, including the basic polymerase 2 (PB2), basic polymerase 1 (PB1), acidic polymerase (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and non-structural protein (NS) segments. The HA gene and the NA gene encode haemagglutinin and neuraminidase, respectively, which are integral membrane proteins. Influenza A viruses are categorized into different subtypes on the basis of antigenic differences in their two surface glycoproteins, HA and NA. Currently, 16 different HA and 9 different NA subtypes of influenza viruses have been detected in avian species (Noda, 2012).

The H4 avian influenza virus (AIV) circulates worldwide and has been detected in wild birds and poultry during normal surveillance conducted in Asian, European, and North American countries (Bui et al., 2012; Deng et al., 2013; Donis et al., 1989; Liang et al., 2016; Wisedchanwet et al., 2011). Certain H4 viruses can directly infect mice directly without prior adaptation and can cause severe respiratory disease, and even death (Bui et al., 2012; Driskell et al., 2010; Liang et al., 2016). In a recent study, Liang et al. showed that multiple genotypes of H4 viruses (including H4N2, H4N6 and H4N8) are co-circulating in the live poultry markets of China and that certain H4 viruses can replicate in mice, possess human-type receptor binding specificity, and be transmitted between guinea pigs via direct contact and respiratory droplets (Liang et al., 2016).

H9N2 subtype influenza viruses have been detected in different species of wild birds and domestic poultry in many countries for several

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decades (Homme et al., 1970; Kawaoka et al., 1988; Li et al., 2005; Li et al., 2014). These viruses have also been detected in pigs and humans (Gou et al., 2000; Guo et al., 1999; Guo et al., 2002; Huang et al., 2015; Peiris et al., 2001; Xu et al., 2004; Yu et al., 2008). H9N2 viruses continue to present challenges to the poultry industry and to human health, not only because they can infect birds and humans but also because they provide partial or whole internal gene segments to emerging reassortants such as the clade 7.2 H5N2 natural reassortants, the recently discovered clade 2.3.4.4 H5N6 viruses, the newly detected H7N7 viruses, and the human-infecting H7N9 and H10N8 viruses initially reported in 2013 (Chen et al., 2014; Li et al., 2014, 2016; Shen et al., 2016; SHI JianZhong1 et al., 2013; Zhao et al., 2012). In our previous study, we found that the H9N2 viruses naturally isolated during the years 2009 to 2013 had acquired a preference for human-type α-2,6 sialic acid receptors and had developed airborne transmissibility in ferrets. Most significantly, H9N2 viruses can function as "vehicles" that deliver different subtypes of influenza viruses from avian species to humans (Li et al., 2014). Although H4 and H9 subtype viruses are classified as low pathogenic avian influenza virus (LPAIV) strains, these viruses are extensively distributed in domestic poultry and wild waterfowl. The inevitable contact of these avian species promotes the circulation or co-circulation of H4 and H9 viruses.

Influenza viruses possess a segmented genome that supports the rapid production of variant progeny influenza viruses through genetic reassortment (Marshall et al., 2013). Genetic reassortment is one of the most important factors in influenza virus evolution, cross-species transmission and generation of antigenically novel isolate generation (Holmes et al., 2005; Rambaut et al., 2008). Theoretically, when two parent influenza viruses that differ in all eight gene segments co-infect the same cell, their genes can reassort to produce 256 new different progeny viruses. In a previous study, Zhang et al. (2013b) systemically created 127 reassortant viruses between duck H5N1 and human-infective pandemic/09 H1N1 by reverse genetics and showed that H5N1 subtype viruses do have the potential to acquire mammalian transmissibility by reassortment (Zhang et al., 2013b). Two other studies used reverse genetics to generate 127 reassortant viruses carrying the HA segment from avian H5N1 in the genetic background of human H3N2 or carrying the HA segment from H9N2 in the genetic background of H1N1; these studies showed that 49% (H5N1/H3N2 reassortant) and 58% (H9N2/H1N1 reassortant) of the reassortant viruses replicated efficiently in mammalian cells (Li et al., 2010a; Sun et al., 2011). Tao et al. used the H3N2 wild-type (*wt*) virus and a mutated variant (var) as parental viruses to co-infect guinea pigs in transmission studies and found that the co-infection achieved supported high levels of reassortment (Tao et al., 2015).

Although several studies have investigated the reassortment of two different influenza strains by reverse genetics or co-infection in cells or animals, the co-infection of AIVs in avian species and the biological properties of the reassortants need further investigation. Herein, we systemically studied the reassortment of two viruses in chickens through co-infection with H4N6 and H9N2 subtype viruses and tested the biological characteristics of the reassortants. Our results indicate that a high reassortment frequency was prevalent in chickens co-infected with H4N6 and H9N2 viruses and that some of the reassortants showed enhanced pathogenicity in mice compared with parental viruses. Therefore, the genetic compatibility of the prevalent H4N6 and H9N2 viruses increased the potential risk to public health.

2. Materials and methods

2.1. Ethics statement

Chicken and mouse studies were performed in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The animal research was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS).

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) cells used for virus titration were cultured in Dulbecco's modified Eagle's medium (CORNING, Cellgro) medium with 4% fetal bovine serum (FBS). All cells were incubated at 37 °C with 5% CO2. These cells were used to isolate plaque clones from tracheal and cloacal samples. To investigate the reassortment of AIVs in chickens, we selected H4N6 and H9N2 viruses that were isolated from chickens in China. Two wild-type H4N6 and H9N2 viruses (A/chicken/Shandong/36/2016 H4N6 and A/chicken/Henan/815/2016 H9N2, GISAID accession numbers EPI1221838-EPI1221845 and EPI1221848-EPI1221855, respectively) were grown in ten-day-old SPF embryonated chicken eggs at 37 °C for 48 h. The allantoic fluid was collected and stored at −70 °C.

2.3. Genetic analysis of H4N6 and H9N2 viruses

Prior to the co-infection study, we genetically characterized the two infectious viruses. H4N6 and H9N2 viral RNA was extracted from virus-infected allantoic fluid with the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed with a set of gene-specific primers (the primer sequences are not shown), and the products were sequenced on an Applied Biosystems DNA analyser. The gene-specific primer sequences are available upon request. The nucleotide sequences were edited by using the Seqman module of the DNAStar package. Sequence alignment of the two viruses was performed with the Align program of the MEGA 7.0 software.

2.4. Co-infection experiments in chickens and RT-PCR detection

Twenty-five six-week-old SPF chickens were divided into five groups (n = 5 per group). All birds were inoculated intranasally with 1×10^6 EID_{50} H4N6 and 1×10^6 EID_{50} H9N2 in a volume of 100 µl. The first two groups (A and B) were first inoculated with H4N6 followed by H9N2 eight or sixteen hours later. The third and fourth groups (C and D) were first inoculated with H9N2 and then with H4N6 eight or sixteen hours later. The fifth group (E) was inoculated with both viruses simultaneously. The chicken tracheal and cloacal swabs from each group were collected in marked tubes daily on days 1–7 post-inoculation and were stored at −70 °C until further analysis. Viral RNA was extracted from the samples of tracheal and cloacal swabs with the QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The gene-specific primers used to identify each segment of the H4N6 and H9N2 viruses were designed by the Oligo 6 software.

RT-PCR was performed with a set of gene-specific primers for each of the eight gene segments (primer sequences are available upon request) to detect each segment of the H4N6 and H9N2 viruses, respectively.

2.5. Isolation reassortants from the tracheal and cloacal samples

According to the RT-PCR detection results, the tracheal or cloacal samples containing the potential reassortants were selected for isolating the pure virus. Briefly, confluent MDCK cells in six-well plates were infected with twofold serial dilutions (1/10-1/1280) of tracheal or cloacal samples and then were overlaid with immunodiffusion grade agarose gel (Sigma-Aldrich). Following at least 60 h of incubation at 37 °C, viruses were harvested from the infection foci and were inoculated into ten-day-old SPF eggs. RT-PCR methods were used to confirm the presence of each segment of H4N6 and H9N2 from the plaque-purified viruses. After plaque purification, the viruses that were pure reassortants were selected for further analysis.