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Genetic analysis and biological characteristics of different internal gene origin H5N6 reassortment avian influenza virus in China in 2016

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ABSTRACT

Clade 2.3.4.4 of H5N6 subtype Avian Influenza Viruses (AIVs) has become dominant clade in South-East Asia. So far, a total of 16 cases of human infection, including 6 deaths, have been confirmed since 2014. In this study, we systematically investigated the genetic evolution and biological characteristics of these viruses. We first carried out phylogenetic and statistical analysis of all H5N6 viruses that were downloaded from Influenza Research Database, GISAID and isolates from our lab. We found that H5N6 AIVs continued to reassort with other AIVs subtypes since 2014. Among these H5N6 reassortments, four main gene types were identified: A (internal genes of H5N1-origin), B (PB2 of H6-origin, and others of H5N1-origin), C (internal genes of H9-origin) and D (PB2 of H6-origin and PB1of H3-origin, and others of H5N1). In addition, after several years of evolution, gene type D is currently the dominant gene type. To systematically compare the genetic and evolutionary characteristics and pathogenicity of these viruses, four H5N6 AIVs of different gene types were selected for further analysis. S4, XZ6, GD1602 and YZ587 virus represented gene type A, B, C and D, respectively. Their NA genes were all originated from H6 and their whole genome showed a high similarity with human isolates. All these isolates could both bind with SA-α2,3 Gal and SA-α2,6 Gal receptors. Pathogenicity test showed that these viruses were highly pathogenic in chickens, while YZ587 showed the lowest virulence. Moreover, XZ6 and S4 viruses were highly pathogenic in ducks and moderately pathogenic in mice, while GD1602 and YZ587 viruses were no-pathogenic in these animals. Interestingly, GD1602 and YZ587-like viruses were responsible for 4 and 2 human infection cases in 2016, respectively. Therefore, our study showed that the YZ587 virus which has mixed internal genes, showed lower virulence in avian species and mammals compared to other genotype viruses. Overall, our findings suggest that the H5N6 avian influenza virus is undergoing constantly evolving and reassortment. Thus, our study highlights the necessary of continued surveillance of the H5N6 AIVs in birds and paying close attention to the spread of these novel reassortment viruses.

1. Introduction

In the process of constant evolution, H5 subtype AIVs of the Clade 2.3.4.4 have been reassorted with various NA subtypes (H5N1, H5N2, H5N6 and H5N8) and have been constantly detected in different domestic poultry in China (Lee et al., 2017a; Li et al., 2017; Moatasim et al., 2017; Zhao et al., 2012). In addition, multiple subtype influenza viruses (H3, H4, H6, H9 and H10), that are considered to be a reassortment source of AIVs in China, have also circulated and evolved in live poultry markets (Liu et al., 2003). Therefore, H5 subtype AIVs are now reassorted with various NA genes, and with various internal genes of low pathogenic AIVs (LPAIVs) (Lee et al., 2017b; Ma et al., 2016; Moatasim et al., 2017; Wu et al., 2017).

Currently, the prevalent clade of the H5 subtype in Asia and South-East Asia is 2.3.4.4 (Bi et al., 2016a). In this clade, the H5N6 AIVs are the major NA subtypes found in Asia and Southeast Asia (OIE: http://www.oie.int/). The clade 2.3.4.4 H5N6 AIVs was initially found in China in 2013, and then it quickly spread to Laos and Vietnam in 2014 and 2015 (Claes et al., 2016). Thus far, H5N6 AIVs are the most prevalent, the most widespread and the most harmful subtypes in Asia and

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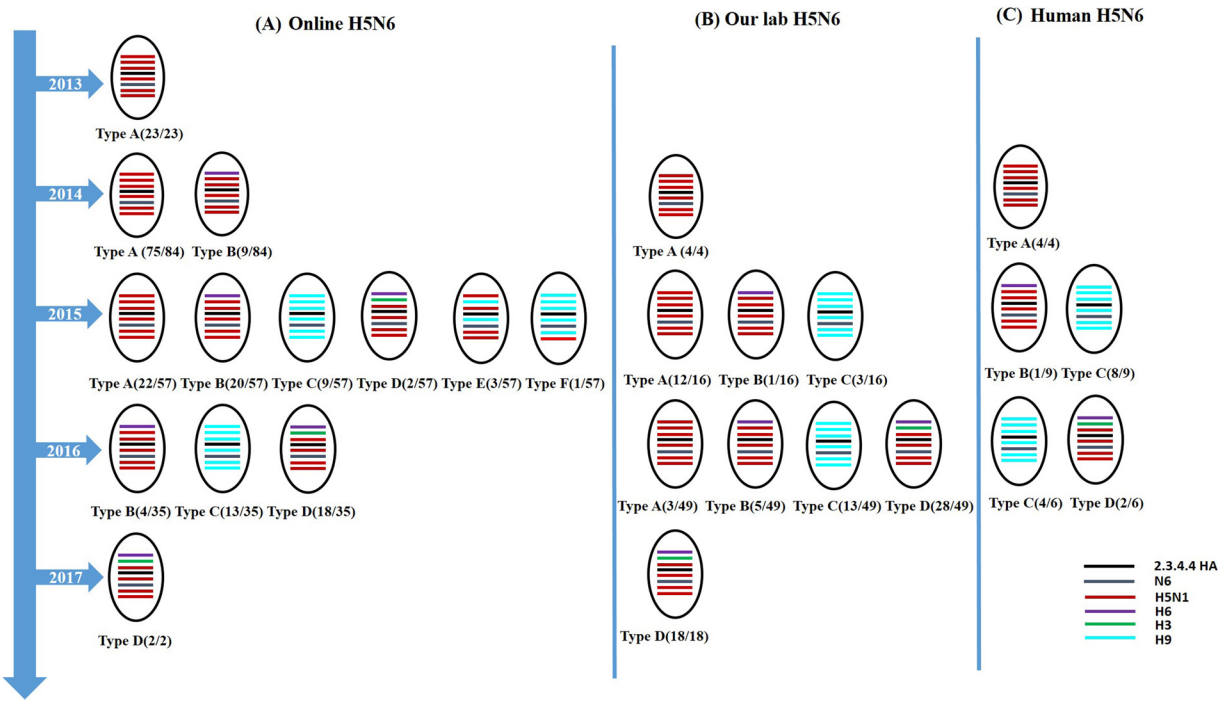

Fig. 1. Internal genes and gene types years distribution of H5N6 AIVs. (A) showed the different gene reassortments years distribution histogram of all online H5N6 AIVs download from Influenza Research Database, NCBI and GISAID. (B) showed the different gene reassortments years distribution histogram of all H5N6 AIVs from Our lab data. (C) showed the different gene reassortments of the human isolates years distribution histogram of all H5N6 AIVs download from GISAID. Type A means all internal genes were H5N1-origin; Type B means all internal genes were H5N1 except PB2 gene was H6; Type C means all internal genes were H9N2-origin; Type D means PB2 gene was H6-origin, PB1 gene was H3-origin and others were H5N1-origin; Type E means PB1 and NP genes were H9N2-origin and others were H5N1-origin; Type F all internal genes were H9N2-origin except NS gene was H5N1-origin.

Southeast Asia (OIE). The first human case of H5N6 AIV infection was reported in Sichuan in China in 2014(Pan et al., 2016). According to World Health Organization (WHO: http://www.who.int/influenza/en/), by December 2017, a total of 19 laboratory-confirmed cases of human infection with H5N6 AIVs, including 6 deaths have occurred in China since 2014. Therefore, the H5N6 subtype AIVs potentially present a substantial threat to both public and animal health.

During the long-term surveillance for AIVs in live poultry markets (LPMs) in eastern China, our lab has found that the H5N6 AIVs have been undergoing constant reassortment (Fig. 1B). Moreover, other studies also have showed that the H5N6 AIVs have continued to reassort with other AIVs subtype since 2014, and various kinds of novel H5N6 reassortments containing different origins of internal genes have been found (Bi et al., 2016a; Okamatsu et al., 2017; Yang et al., 2017a). In this study, to systematically investigate the phylogenies and biological characteristics of these reassortments, we analyzed all the available H5N6 viruses in our labs, Influenza Research Database and GISAID. In addition, we found that there are six main gene types during 2013–2017, among of them, four of which was the main prevalent gene type according to our lab data. Therefore, in order to systematically compare the biological characteristic of these gene types viruses, we selected four representative variants which carrying different internal genes in 2016 for further analysis of phylogenetic and molecular characteristics, pathogenicity (in chickens, ducks and mice), receptor-binding properties and antigenic analyses. Our study further accelerates our knowledge of the genetic evolution and pathogenicity characteristics of these viruses in avian and mammalian hosts.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations 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 protocols for animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (approval number: SYXK-SU-2007-0005), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals. All experiments involving live viruses and animals were housed in negative-pressure isolators with HEPA filters in bio-safety level 3 (BSL3) animal facilities in accordance with the institutional bio-safety manual.

2.2. Sample collection

To monitor the dynamic changes in avian influenza virus, and to assess the current risk of influenza virus to public health, our laboratory has conducted regular sampling of the LPMs in East China. We mainly collected cloacal and throat swabs from healthy flocks from the LPMs in Jiangsu province (Yangzhou LPM, Zhenjiang LPM, Jiangyin LPM and so on) since the outbreak of AIVs is serious in this area and received samples from agriculture committee of East China. Therefore, we can monitor the vast majority regions of East China. During January 2016 to August 2017, we have collected 1724 cloacal and throat swab samples. Subsequently, fresh swab samples were transferred into a 1.5 ml tube containing Phosphate Buffered Saline (PBS) mixed with four kinds of antibiotics (Penicillin, streptomycin, kanamycin and gentamicin) and then these samples were stored in the refrigerator at −70 °C for future analysis.

2.3. Virus isolation and identification

All samples were repeatedly frozen and melted for three times and then the swab was squeezed out of the tube. Samples were then centrifuged at 8000 rpm/min, and the total genome was extracted from the